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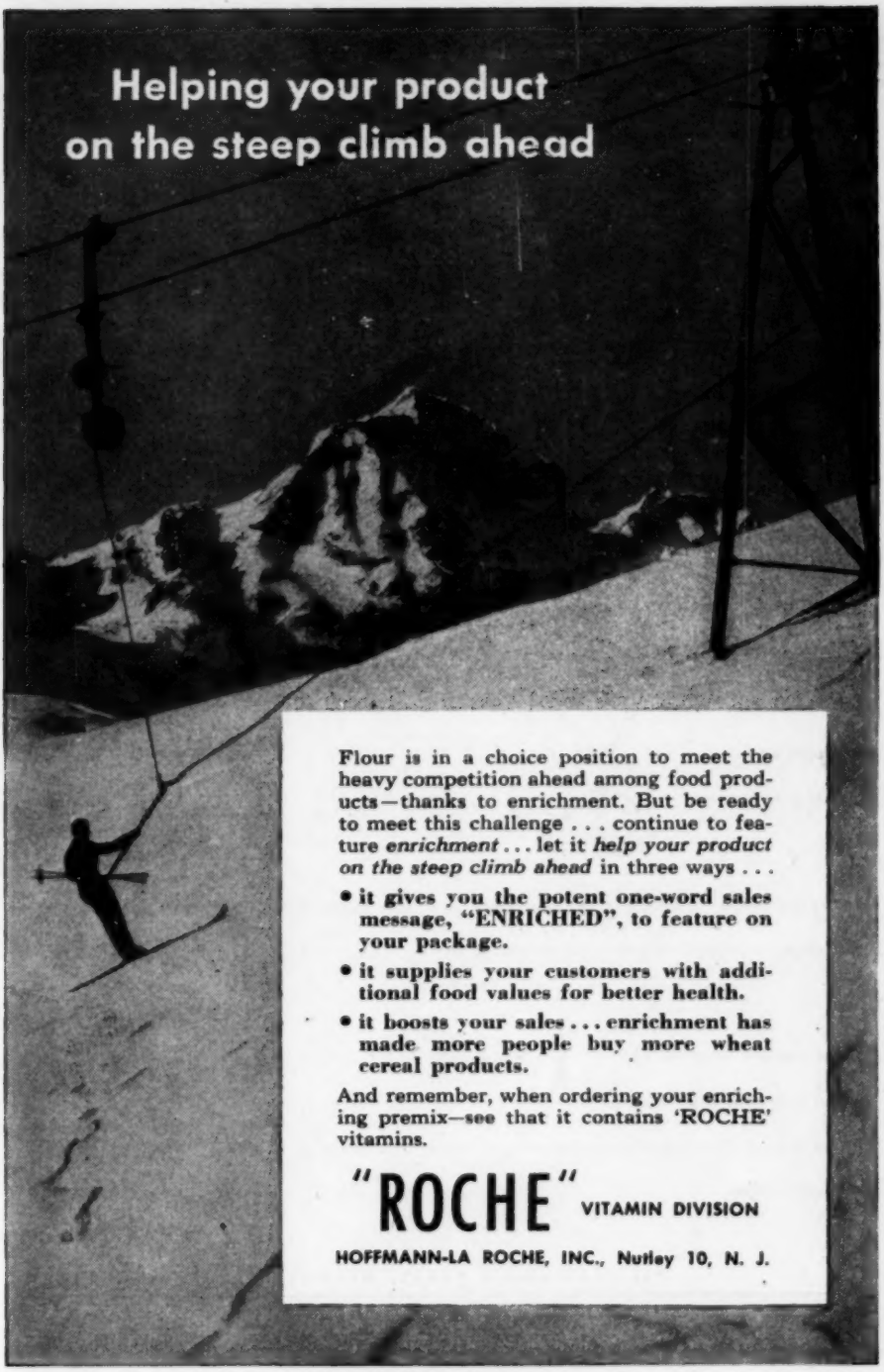

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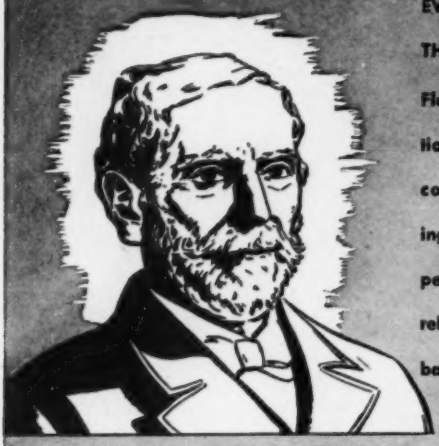

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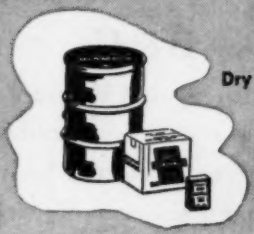

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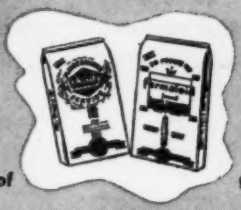

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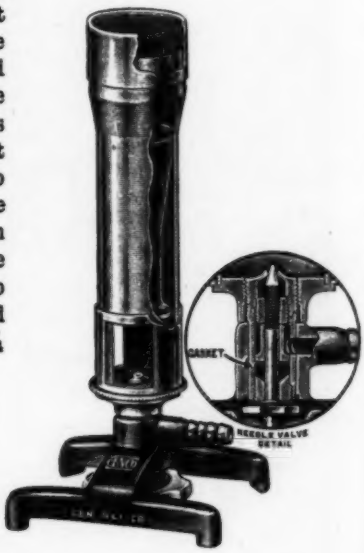

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CEREAL CHEMISTRY

VOL. XXIII MARCH, 1946 No. 2

A MACHINE FOR MEASURING THE EXTENSIBILITY AND RESISTANCE TO EXTENSION OF GLUTEN [1]

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Grain Research Laboratory, Board of Grain Commissioners for Canada, Winnipeg, Manitoba

(Received for publication September 20, 1945)

The physical properties of gluten are important in breadmaking and have been widely studied, but there is as yet no entirely satisfactory and generally accepted method of measuring them. It is true that any experienced cereal chemist can make an excellent estimate of the quality of the gluten washed from a dough by manipulating it in his hands; the difficulties are that the judgment is subjective and depends on the experience of the chemist, and that the resulting opinion cannot be readily recorded or transmitted to another person. There have been a number of attempts to overcome these difficulties by devising machines for measuring certain properties of gluten. These machines and related ones for measuring the physical properties of dough have been reviewed by Bailey (1940), and two other machines have since been reported.

Kress (1924) described one of the earliest machines for testing gluten and James and Huber (1927) used it for measuring the properties of glutens washed from different samples and subjected to various treatments. Mohs, Schmidt, and Frank (1939) reported a technique for use with the Brabender glutograph, which draws a "glutogram" representing extension of the gluten and the force required to extend it until it breaks. A device for measuring the flow and spring of crude gluten was described by Baker, Parker, and Mize (1942); and Baker, Mize, and Parker (1943) presented data obtained with a machine which measured the force necessary to penetrate a gluten ball held in a special holder.

The apparatus described in this paper measures the extensibility of crude gluten and its resistance to extension. It has been called a

[1] Published as paper No. 80 of the Grain Research Laboratory, Board of Grain Commissioners for Canada, Winnipeg, and as paper No. 243 of the Associate Committee on Grain Research (Canada).

"Stretchometer." The design presents nothing fundamentally new since it is similar in many respects to the Brabender glutograph and to some other apparatus. However, for various reasons it seemed advisable to design and build a new machine rather than make a close copy of an old one. This paper reports on the development of a standard technique for use with the Stretchometer, on the application of the machine to studies of gluten quality, and on the limitations encountered in these studies.

Apparatus and Methods

The Stretchometer. A photograph of the Stretchometer is shown in Figure 1. It consists of a spring balance (covered by a face-plate *A*)

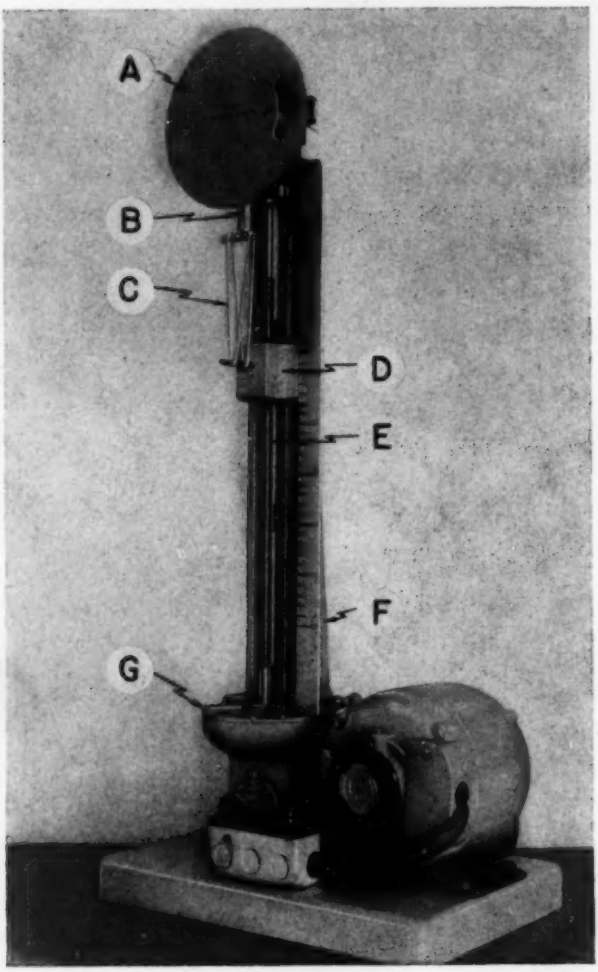

Fig. 1. Gluten Stretchometer.

supported at the top of a vertical frame *F* on which a block *D* is moved by the screw *E*, which is driven by a motor operating through the gear box *G*.

A ring of gluten is hung over an inverted Tee *B* suspended from the balance, and a removable pin is passed through the two loops of gluten *C*, and inserted into the block *D*. When the motor is started, the four strands of gluten are stretched simultaneously. The distance that the gluten stretches before breaking is noted from the position of the pointer on the vertical scale on the right, and the resistance to extension is measured by the balance. The vertical scale is graduated from 0 to 40 cm, and the balance from 0 to 150 g.

A drawing of the balance assembly at the top of the Stretchometer is shown in Figure 2. The balance is made from a clock spring, with its inner end fastened to a spindle mounted on ball bearings, and the

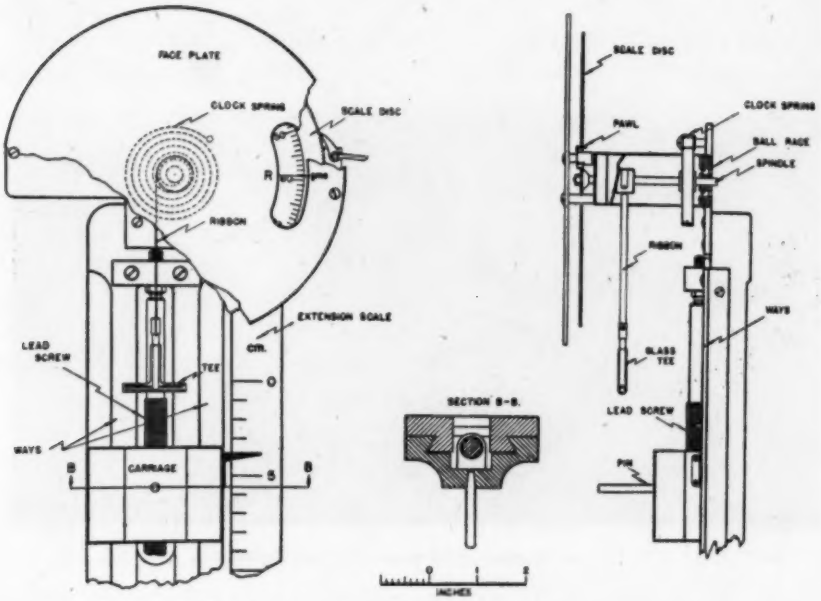

Fig. 2. Partial detail of Stretchometer assembly.

outer end attached to the frame. The glass Tee, on which the gluten is hung, is attached to a thin metal band wound around a one-half inch boss on the spindle. An aluminum disc attached to the front end of the spindle has a scale calibrated in grams, and a pawl engaging with notches on the edge of the disc holds it in the maximum position after the weight is removed when the gluten breaks. Readings are made through a window in the face plate.

The main stand was cast in bronze from a pattern made in the laboratory, and the ways and other parts were subsequently machined. The base of the stand was designed to fit the top of the inverted gear box *G*, salvaged from an old Hobart dough mixer, which serves as a reducing gear. The operating screw *E* is set into the vertical shaft in the gear box and is held at the top by a pivot thrust bearing. It has 20 threads to the inch and is so made that the block carriage *D* can run off the thread at the top to set itself automatically at zero, and, as a safety device, can also run off at the bottom. The machine is driven by a reversible motor so that the block can be returned to the starting position after each test. Motor and V-belt drive were so selected that the block moves at 40 cm per minute when the gear box lever is in the "low" position.

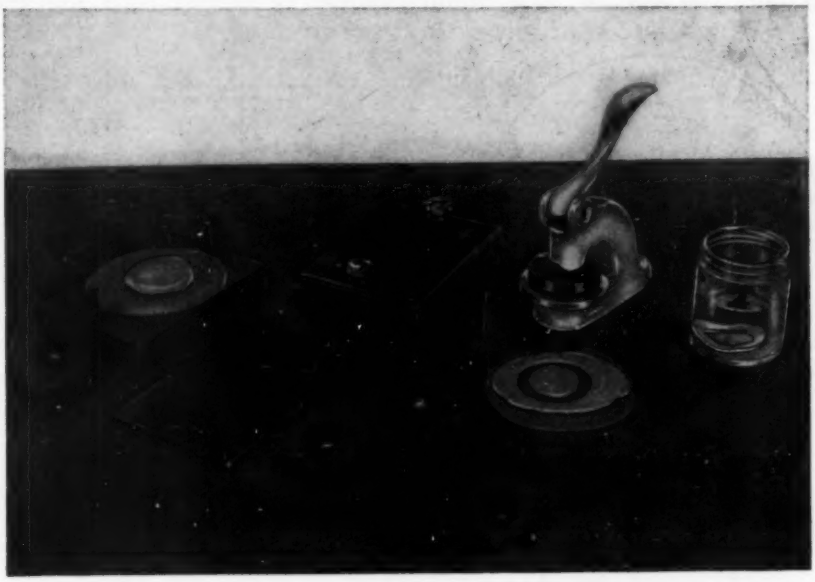

Fig. 3. Equipment for preparing rings of gluten.

Other Equipment. For preparing the rings of gluten, pairs of iron plates for freezing gluten discs, and a cutter for cutting the rings were made. The plate assemblies, both open and closed, are shown on the left in Figure 3, and the cutter is shown at the right. The plates are $4 \times 5\frac{1}{2}$ inches and are made from 3/16-inch iron. Spacing studs on the lower plate, against which the top plate comes to rest, are so adjusted that a gluten disc with a uniform thickness of 3 mm is produced. The cutter is made from an old embossing stamp and cuts out the rings in one operation. The ring has external and internal diameter of 50 and 35 mm and weighs about 3 g.

Preparation of Sample. The technique finally adopted for use with the Stretchometer is briefly described in this subsection, and the experiments dealing with the effects of varying the conditions for different steps of the technique are described later. For duplicate tests, a dough of baking absorption is prepared by mixing 75 g of flour with water for one minute in the Hobart-Swanson mixer. In experiments made so far, the gluten has been washed out by hand under a small stream of tap water maintained at 25°C. The gluten is divided into two equal portions, which are rounded into balls. Each ball is then placed between a pair of cellulose acetate discs, 0.01 inch thick, and squeezed into a disc by clamping between the plates described above. The gluten is then frozen by placing the plate assembly in a chamber at −20°C for 45 minutes. This is done to facilitate the preparation of the test rings. To accelerate freezing, the plate assemblies are kept in the refrigerator when not in use and are thus precooled.

When the frozen sample is removed from the plates, the cellulose acetate discs peel off readily and the gluten remains frozen long enough for the ring to be cut from it. Before stretching, the rings are conditioned for 30 minutes in water in a small jar placed in a constant temperature water bath at 25°C. Fifty milliliters of water at 30°C are put into the jar with the frozen gluten and the conditioning temperature of 25°C is thus reached almost immediately.

Stretching. The method of making the stretching test will be obvious from the foregoing descriptions of the Stretchometer. Extensibility is measured in centimeters by means of the longitudinal scale, and resistance to extension is measured in grams by the balance.

Other Methods. Fisher and Halton (1936) described two moisture methods for gluten; one involved boiling the gluten before drying in an air oven, and the second made use of specific gravity. Another method has been devised in this laboratory. The surface moisture of the sample tested on the Stretchometer is removed by manipulating it in the hands, and the sample is placed in a 25-ml crucible, covered with a watch glass, and weighed. Enough 95% ethyl alcohol (4 ml) is added to cover the sample and after a few minutes the uncovered crucible is put in an air oven at 130°C. The alcohol causes the gluten to puff up during drying as it does in the vacuum oven, and complete drying is obtained in 3 hours. This method gives the same result and replicability as the vacuum oven method.

The dried samples can be readily crushed into fine powder with a pestle while in the crucible. After redrying the crushed samples for 30 minutes, aliquots of 0.2 g are weighed for Kjeldahl determinations.

Standardization of the Test Procedure

In developing a procedure for use with a new machine, such as the Stretchometer, a number of arbitrary decisions must first be made on the conditions under which each step of the procedure shall be carried out. A large amount of investigation of the effects of varying the conditions and on the precision with which the conditions should be controlled can then be undertaken; but if this is too extensive, the more important task of applying the test is delayed. A compromise must be effected, and in the present instance this involved making limited studies of the conditions for several of the major steps of the procedure. The results are described in the following subsections.

Mixing of Dough. Information was sought on the possible effect on Stretchometer results of the amount of work done on the gluten during mixing. Accordingly, doughs from three different flours were prepared by mixing for 1 and 3 minutes in a Hobart-Swanson mixer, and glutens were washed out and tested. Mean values for eight replications are as follows:

Flour	Gluten properties							
	Moisture content		Protein content		Resistance		Extensibility	
	1 min	3 min	1 min	3 min	1 min	3 min	1 min	3 min
	%	%	%	%	*g*	*g*	*cm*	*cm*
1	66.3	67.9	80.4	81.0	67	70	26	26
2	65.8	66.9	81.0	81.4	93	93	24	24
3	65.4	67.0	81.4	80.5	98	96	22	24

The only constant difference between glutens from doughs mixed 1 and 3 minutes is that the latter have higher moisture contents, that is, they showed greater absorptions. But this difference did not affect either extensibility or resistance. Glutens of similar mean protein content were readily obtained irrespective of whether the doughs were mixed for 1 or 3 minutes, and even the small differences in protein content that exist are reflected by parallel differences in the resistance values.

As a smooth dough can be obtained by mixing for 1 minute, this time was selected as standard. Longer mixing, particularly with weaker flours, makes the dough more difficult to handle.

Amount of Washing. A number of studies were made of the effects on gluten of the amount of washing and manipulation it receives while it is being recovered from the dough. The results of studies made with two different flours are shown in Figure 4. Sample 1 was an experimentally milled flour of 12.6% protein, while sample 2 was a

commercially milled flour of a similar protein content of 12.7%. Replicate doughs from each flour were washed by one operator for different lengths of time in random order. Washing time was varied between 6 and 32 minutes.

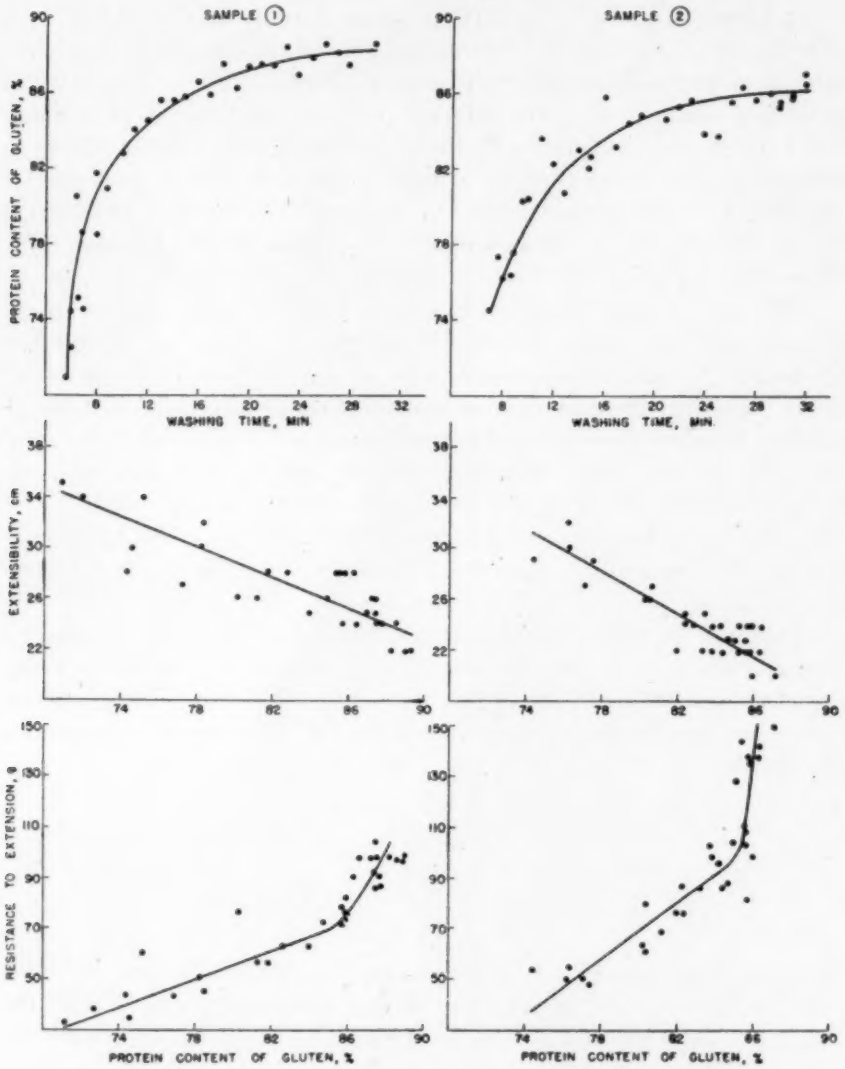

Fig. 4. Relations illustrating effects of washing glutens for different lengths of time.

Relations between washing time and the protein content of the glutens are shown in the top pair of curves. The curves have the expected shape; the protein content of the crude gluten increased rapidly

during the early stages of washing and less rapidly as washing progressed. Sample 1 apparently yielded its gluten more readily; it attained a higher final protein content and approached this level more rapidly. The curves also show that Sample 2 was more difficult to handle uniformly during washing.

It seemed best to accept the protein content of the gluten as a criterion of the amount of washing, and the middle pair of curves therefore show extensibility plotted against protein content rather than against washing time. The relation between the amount of washing and extensibility appears to be linear, and differences of about 10 cm in the extensibility of the gluten from a given sample of flour can be obtained by wide variations in the amount of washing. Deviations of the points from the regression lines are almost wholly accounted for by the experimental error, which suggests necessity for replication.

The bottom pair of curves in Figure 4 deal with resistance to extension, and here again the deviations from the curve are accounted for mainly by the experimental error of the Stretchometer measurement. During the first part of the washing period there is a linear relation between protein content and resistance, but towards the end, at about 85% protein, the curve rises sharply so that resistance increases while the protein content of the gluten changes little. For that reason further experiments described in this paper deal with glutens washed to protein contents within the range in which relations are linear.

Freezing. Rapid freezing seemed desirable, and as the lowest temperature that could be conveniently maintained in the freezing cabinet was −20°C, this temperature was adopted for all tests. Mean values for quadruplicate discs frozen for 20, 40, and 60 minutes are given below:

Time	Extensibility	Resistance
min	*cm*	*g*
20	25.0	73
40	29.5	78
60	30.0	75

At 20 minutes the gluten did not appear to be fully frozen. Differences between the results for 40 and 60 minutes are within the experimental error and a time of 45 minutes was chosen mainly to suit the testing schedule.

Conditioning Temperature. The influence of the temperature at which the gluten rings are conditioned after freezing was studied by testing duplicate rings from five glutens conditioned for 30 minutes at each of three temperatures of 20°, 25°, and 30°C.

Temperature °C	Extensibility *cm*	Resistance *g*	Moisture in gluten %
20	25.1	100	65.8
25	25.4	81	65.8
30	26.1	77	66.0

The mean results show that increasing the temperature increased the extensibility slightly and reduced the resistance to extension. A temperature of 25°C was chosen as standard, and it was obvious that temperature would have to be closely controlled by means of a water bath and thermostat.

Conditioning Time. Duplicate tests were made on five glutens conditioned at 25°C for 20, 40, and 60 minutes.

Time *min*	Extensibility *cm*	Resistance *g*	Moisture in gluten %
20	25.8	89	65.9
40	27.4	87	66.1
60	25.0	80	66.3

Erratic results were obtained for extensibility, but increasing conditioning time reduced the resistance to extension and increased the moisture content of the gluten. These effects are not large and minor errors in timing would not be serious. A conditioning time of 30 minutes was selected as standard.

Uniformity of Sample Rings. The suitability of the cutting technique and the degree of uniformity required in the sample rings was investigated by exaggerating the differences. Rings of different weight were obtained by cutting them from glutens frozen in different thicknesses. Data for the means of triplicate tests are given below.

Weight of ring *g*	Extensibility *cm*	Resistance *g*
2.87	29	63
2.94	30	68
3.04	30	68
3.20	29	73

Extensibility is little affected by increasing the weight of the ring, but resistance increases appreciably. In later experiments, however, it was shown that the standard error for the weight of a single ring is only about ± 0.03 g, which is not large enough to have a serious effect on resistance. It appeared that gluten rings weighing about 3 g would be suitable, and space adjustments between plates were thus left at 3 mm.

Replicability of Tests. Statistics on the precision of the Stretchometer and auxiliary tests were obtained from three major experiments. Standard errors of single determinations, calculated from differences between tests made on duplicate rings, are given in Table I.

TABLE I

PRECISION OF STRETCHOMETER AND AUXILIARY TESTS

Property of gluten	Experiment 1 (22 pairs)	Experiment 2 (48 pairs)	Experiment 3 (96 pairs)
Standard errors of single determinations			
Weight of sample ring, g	0.02	0.04	0.03
Moisture content of gluten, %	0.18	0.19	0.18
Protein content of gluten, %	0.35	0.39	0.38
Extensibility, cm	1.4	2.0	2.2
Resistance, g	4.7	8.0	5.0
Coefficients of variability			
Extensibility, %	5.1	8.2	6.9
Resistance, %	7.7	9.3	8.2

Standard errors for weight of rings, moisture, and protein are less than 1%, and are very satisfactory. On the other hand, standard errors for the Stretchometer values are somewhat higher. Differences in the magnitude of the errors of extensibility and resistance measurements for the three experiments are not fully explained by differences in the mean values. This is demonstrated by the coefficients of variability given in the last two lines of the table, which are not much more uniform than the corresponding standard errors.

The failure to obtain better replicability for the Stretchometer measurements is undoubtedly due to the difficulty of preparing duplicate gluten rings that will invariably stretch and break in the same manner. If quadruplicate tests are made, the standard error is reduced by half (i.e., divided by $\sqrt{4}$), and with such replication differences of about 4% in extensibility and resistance can be demonstrated. In practice it has been found that the precision obtained with duplicate tests is adequate for most investigations, because the principal experimental error is in preparing the gluten rather than in testing it.

Application of the Stretchometer to Studies of Gluten Quality

The Stretchometer has been used in studies of differences in gluten quality among a series of samples of Thatcher wheat and among a second series consisting of composite samples of six different varieties. The results throw some light on the inter- and intravarietal relations between Stretchometer measurements and other quality data, and also serve to emphasize some of the difficulties that must be overcome before the Stretchometer can be used as a routine rather than as a research tool.

Thatcher Series. Eight samples of flour, milled from Thatcher wheat, and ranging in protein content from 10.5 to 16.5%, were obtained. For each sample of flour, a series of 12 glutens of increasing protein content were prepared by different amounts of washing. The

amount of washing required was judged by weighing the gluten mass from time to time until a predetermined weight was obtained. Extensibility, resistance to extension, moisture content, and protein content were determined for each gluten.

The detailed data for the Stretchometer measurements are shown in Figure 5, in which both extensibility and resistance are plotted against the protein content of the glutens. There are scatter diagrams for each of the eight samples, and one graph in which all the regression lines have been assembled. The scales on the left deal with resistance, as do the black dots and the lower regression lines. The scales on the right, the open circles, and the upper lines deal with extensibility.

The pattern of the black dots in each diagram shows that there is a close relationship between resistance and the extent to which the gluten is washed, as indicated by the protein content of the gluten (cf. Figure 4). The correlation coefficients lie between .82 and .96, with .71 required for a 1% level of significance. The regression coefficients differ considerably. Values for samples 1, 2, and 3 are about 3.9 g for each per cent of protein, whereas those for samples 5, 6, 7, and 8 are about 2.2 g, and the regression coefficient for sample 4 is much lower, 1.6 g. An analysis of residual variance showed that the differences between the regression coefficients were highly significant (found, $F = 4.7$; required for 1% point, $F = 2.9$). The investigation thus shows that the resistance values for glutens from different samples of the same variety do not change by equal amounts when washing produces equal changes in the protein contents of the glutens.

The analysis of residual variance also showed highly significant differences between centroids ($F = 9.4$), that is, between the mean values for resistance. The lower right-hand diagram in Figure 5 certainly shows that samples 1 and 2 have higher resistances than all other samples with the possible exception of sample 4. Indeed, the only sample whose position with respect to the others cannot be determined with certainty is sample 3; but even its resistance is clearly lower than those of samples 1 and 2 and greater than those of samples 6, 7, and 8. On the whole, therefore, the results are quite clear-cut.

The data on extensibility cannot be interpreted as readily as those on resistance. Most of the correlation coefficients for extensibility and protein content were not significant owing to the small change in extensibility for each per cent protein and the somewhat large error in the determination of extensibility. There is considerable crossing among the regression lines, and the analysis of residual variance did not show significant differences between the slopes. Accordingly, it cannot be assumed that the extensibilities of glutens from different samples change at different rates during washing. On the other hand,

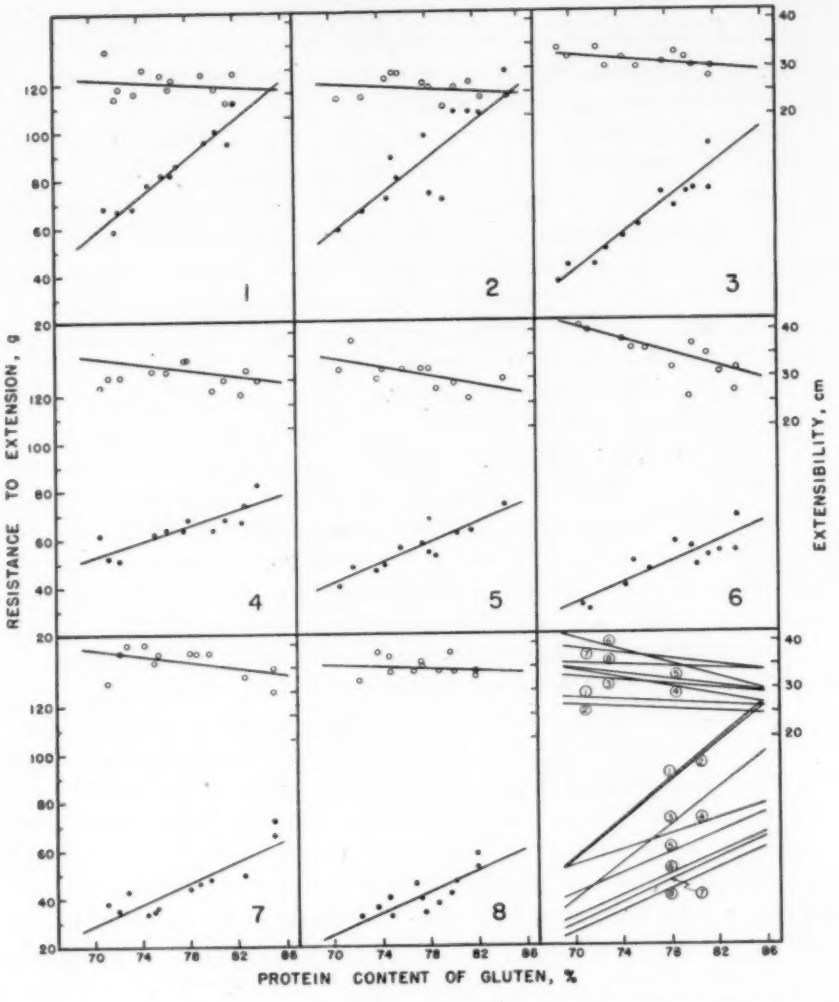

Fig. 5. Relations between protein content of gluten and resistance to extension, and between protein content and extensibility.

significant differences between centroids, or mean values, were established ($F = 26.9$). Reference to the upper group of lines in the assembly diagram in Figure 5 will show that there is little doubt that samples 6, 7, and 8 have higher values than samples 3, 4, and 5, and that the latter have higher values than samples 1 and 2. But within each of these groups the samples cannot be differentiated.

In order to examine possible relations between Stretchometer and other values, data on protein content, absorption, and loaf volume by the malt-phosphate-bromate procedure (Geddes, Aitken, and Fisher,

1940) were obtained for the original flours, and Stretchometer values were calculated to a common protein basis. The mean protein content for all glutens from all flours was 77%; and with the aid of appropriate regression coefficients Stretchometer values for each flour sample were calculated for this protein level. These values are reported in Table II together with gluten moistures, mean protein content of the gluten, and baking data.

TABLE II

STRETCHOMETER AND OTHER DATA FOR THATCHER SERIES

Sample no.	Gluten			Flour		
	Resistance	Extensibility	Moisture	Absorption	Protein	Loaf volume
	g	*cm*	*g*	%	%	*cc*
1	85	28	65.6	60.3	11.8	733
2	84	26	64.9	60.2	13.5	757
3	65	32	65.1	65.6	10.5	630
4	64	32	66.3	62.7	14.6	795
5	56	31	66.4	62.3	16.5	843
6	47	34	67.3	63.7	12.7	796
7	44	36	67.2	63.5	15.1	842
8	40	35	67.7	63.7	16.0	1027

The first two columns of data show that within these samples of one variety there is an inverse relation between resistance and extensibility ($r = -.95$; 1% point is .83). There is also evidence of an inverse relation between resistance and the moisture content of the glutens ($r = -.90$), and, of course, of a direct relation between the latter and extensibility ($r = +.86$). Neither resistance nor extensibility is significantly related to either the protein content or the loaf volume of the flour; and the anomalous absorption for sample 3 interferes with the possible relation between the moisture content of the gluten and the absorption of the flour. Within this series of Thatcher flours, loaf volume appears to depend mainly on the protein content of the flour ($r = .83$). The interpretation of differences between samples as shown by Stretchometer values in Table II is the same as that discussed in dealing with Figure 5.

Intervarietal Series. Stretchometer tests were also made on composite samples of Thatcher, Marquis, and four new varieties (C.T. 141, 149, 508, and 807), representing equal parts of grain from a series of replicated plots grown at six stations in western Canada in 1944. As these samples had been studied as part of another investigation, data were available on baking qualities and on the dough properties measured by the farinograph, extensograph, alveograph, and mixograph. In the present study, the gluten from each sample was tested at nine

levels of protein content obtained by the method described in the preceding section. Regression lines for extensibility and resistance, each plotted against protein content, are shown in Figure 6. The correla-

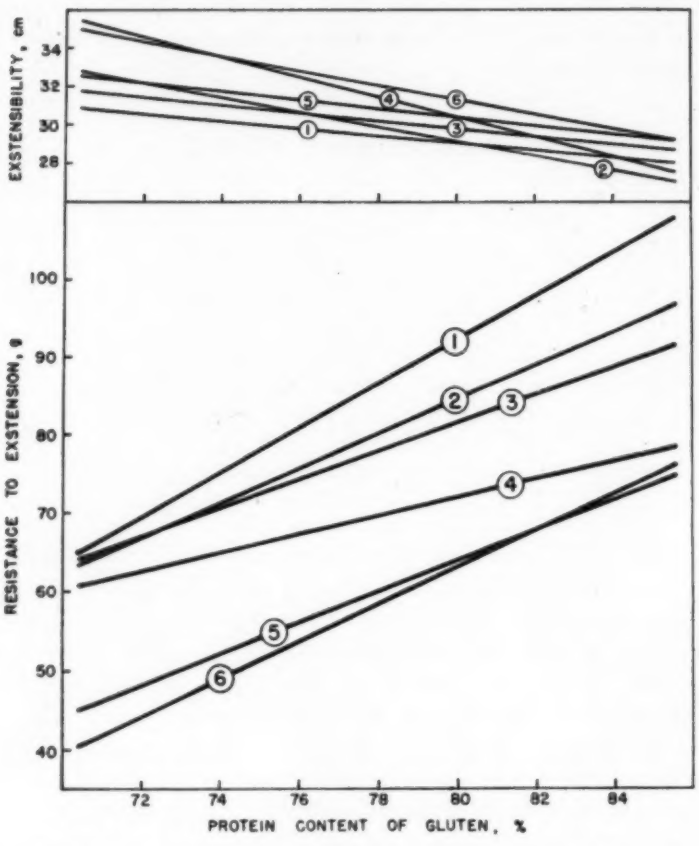

Fig. 6. Lines for six varieties showing regression of Stretchometer values on protein content.

tion coefficients for extensibility and protein were not significant, and an analysis of residual variance did not show significant differences either between regressions or centroids. Thus the study failed to demonstrate differences among extensibilities of the glutens from the six varieties.

Correlation coefficients for resistance and protein content of the gluten were .70, .75, .87, .80, .92, and .87, with .67 and .80 required for 5 and 1% levels of significance; and the regression coefficients were: 2.86, 2.21, 1.77, 1.10, 1.88, and 2.38. Statistical analysis showed no significant differences between regressions but a highly significant

TABLE III

STRETCHOMETER AND BAKING DATA FOR VARIETAL SERIES

Variety	Gluten		Flour	
	Resistance	Extensibility	Flour protein	Loaf volume
	g	*cm*	%	*cc*
1. C. T. 508	88	27	14.2	805
2. Thatcher	82	28	13.6	800
3. C. T. 141	79	28	12.5	735
4. Marquis	70	29	13.0	770
5. C. T. 149	61	29	13.3	780
6. C. T. 807	60	30	13.4	760

difference between centroids ($F = 22.9$; required for 1% level, 3.5). This result would be expected from consideration of Figure 6.

The mean values for the Stretchometer readings were calculated to a common basis of 78.6% protein, which was the general mean for all glutens in this study. The adjusted means, together with data for the protein contents of the original flours and for loaf volume, are given in Table III. In view of the inverse correlation between resistance and extensibility that occurred in the Thatcher series, the corresponding relation shown in Table III is interesting, although no great importance can be attached to it because of the lack of significant differences between extensibility values. There is little evidence of any relation between Stretchometer values and protein content of the flour or loaf volume. In this series again, loaf volume appears to depend mainly on the quantity of protein in the flour.

Figure 7 shows the curves obtained with various machines used for measuring the physical properties of doughs. Normal and high-speed farinograms are shown in the first column, and these are followed by mixograms, extensograms, and alveograms. This figure was prepared for a report which was completed before the Stretchometer study was undertaken. It is therefore of particular interest that the varieties are arranged in exactly the same order as that of Table III, namely, in decreasing order of Stretchometer resistance. Figure 7 shows that this is also the order in which both the farinogram and the mixogram place the varieties with respect to strength. It thus appears that the mixing properties of the doughs are related to the mean resistance values of the glutens.

Resistance to extension is measured for the doughs by the height of the extensogram, while the initial stiffness of the doughs is measured by the height of the alveogram. It might thus be expected that these measurements would be related, at least in part, to the Stretch-

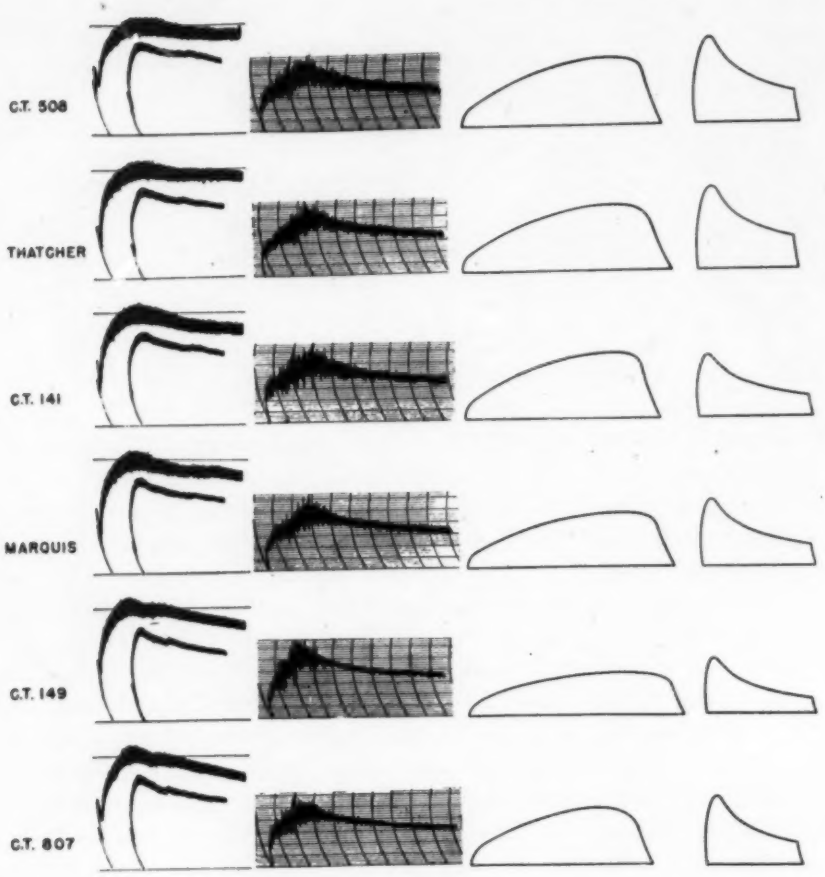

Fig. 7. Normal and high speed farinograms, mixograms, extensograms, and alveograms, for each of six varieties.

ometer resistance. Such a relation appears to exist for the first five varieties, but is upset by the values for the sixth. Since the Stretchometer failed to differentiate between the extensibilities of the varieties it is hardly surprising that no relation is apparent between the Stretchometer value and extensibility as shown by the lengths of the extensogram or alveogram.

General Discussion

The design of the Stretchometer appears to be very satisfactory and no trouble has been experienced in operating it. However, tests made with glutens reconstituted from dried material, many of which have much greater resistance to extension than freshly prepared gluten, indicate the advisability of providing a balance of larger capacity if a second model were built; though this difficulty can be overcome with

the present model by hanging the gluten ring over the Tee in such a way that two rather than four strands of gluten are stretched. Work with rings of dough also suggests that it might be advisable to provide a longer shaft and scale to handle samples which have extensibilities beyond the capacity of the present machine.

When the Stretchometer was built, consideration was given to the advisability of incorporating a recording mechanism so that a curve representing the relation between resistance and stretching time could be drawn. This was not done because it seems to us that the use of a recording mechanism tempts the investigator to undertake complex studies of curve shape, area under the curve, and other characteristics, which are likely to prove fruitless until the significance of the simpler and more readily interpreted measurements of extensibility and resistance to extension is understood.

The technique developed in preparing gluten rings of standard dimensions is simple and reproducible within the limits of sampling error. As freezing can be done on the coils of an ordinary refrigerator, no special equipment is required other than the freezing plates and the ring cutter, and these can be made quite cheaply. The comparable technique used by Mohs, Schmidt, and Frank (1939) with the glutograph involves pretreatment ("homogenization") of the gluten ring which is formed under 25 kg of pressure, in a press designed for the purpose, and held for 30 minutes at 40°C. Before stretching, the ring is cooled for 20 minutes at 18°C. James and Huber (1927) were apparently able to press a satisfactory disc in the Kress (1924) machine after pretreating the gluten by allowing it to stand in water for 10 to 12 hours.

The effect of freezing on gluten properties cannot be readily determined because of the difficulty of preparing rings from unfrozen gluten similar to those made from the frozen gluten. It seems dangerous to assume that this effect will be negligible, although it is probable that it will not be greater than the effects of heating or of allowing the gluten to stand in water for a long period. Nevertheless, the freezing technique must be classed as an arbitrary one adopted to facilitate the test.

The paramount difficulty of comparing the qualities of the gluten in different samples of flour lies in isolating the glutens. It would be ideal if the gluten could be readily obtained in unchanged and "pure" condition, that is, with maximum protein content. But the protein determination takes too long, and there are no other satisfactory criteria for determining when the gluten has been obtained in sufficiently pure form or whether its properties have been changed during extraction. It may well be impossible to obtain pure and unchanged gluten.

The curves in Figure 4, dealing with the effect of continued washing, illustrate the difficulties. Both extensibility and resistance change as the gluten becomes successively purer, that is, as its protein content increases. But the curve for resistance also suggests, by the change in slope towards the end of the washing period, that a stage is reached at about 85% protein when further washing removes little additional nonprotein matter, but continues to increase resistance. And it seems reasonable to suppose that this further change in resistance is caused by the work done during the manipulation of the gluten mass. If this is true, then the question arises as to how much of the increase in resistance (or decrease in extensibility) during the earlier stages of washing results from purification of the gluten and how much results merely from the work done on it.

It might be reasonable to test all glutens at equal but not necessarily maximum protein contents. But here again there are difficulties. Glutens from different flours cannot be brought to the same protein content by washing for equal times since the starch is not released with uniform ease. Accordingly, washing by machine as practiced by Fisher and Halton (1936) would be no great help. Moreover, because different glutens hold different amounts of water, equal final protein contents cannot be obtained by washing to calculated final wet weights. This difficulty could be overcome by pilot tests, though these would be cumbersome. But if all glutens were washed to the same protein content by varying the amount of washing, the work done on the glutens would not be equal and each would probably experience a different amount of change in its physical properties.

These points, which do not appear to have been brought to light in earlier studies, were demonstrated in the present investigation by making comparisons at several different protein contents obtained by washing for different lengths of time. While this technique is by no means ideal, and would hardly prove suitable for a routine test, it does serve to replicate the experiment, to permit comparisons by calculation at a standard final protein content, and to provide additional information on the changes in gluten properties that take place during washing.

Glutens appear to respond differentially to continued washing, and a knowledge of the rate of change of physical properties as washing progresses may be of considerable value. If these changes are brought about partly by the work done on the gluten, they may well be similar to changes that take place in the gluten when a dough is mixed and subjected to other mechanical action during the remainder of the baking procedure. Whether relations of this sort can be demonstrated remains to be seen. In the meantime it seems clear that the differen-

tial response of glutens to washing is caused by differences in their physical properties, and that the elaboration of a suitable technique for comparing glutens is no easy task. However, the difficulties created by the differential effects of washing can easily be overemphasized. Changes in the rank order of samples when the testing procedure is varied are a matter of universal experience. It is well known, for instance, that the placing of a series of flours is frequently changed when the baking formula is changed. But if two samples of flour differ widely in baking quality, then this difference will be apparent by all baking methods. Accordingly, a simplified and standardized method for isolating and testing glutens may well prove useful. It should serve to differentiate glutens that differ appreciably in quality, though it may not serve to separate glutens of somewhat similar quality.

The results of the present study seem sufficiently encouraging to warrant further investigation. Standardization of the washing method, by making this more mechanical, is obviously required, and some improvement in the reproducibility of the Stretchometer measurements is desirable. If these can be achieved it should be possible to make considerable progress in studying gluten quality. Whether progress can be made in relating the physical properties of glutens to the baking qualities of flour remains to be seen. One thing seems certain, however; simple relations between the various properties are hardly to be expected, and the need for large scale studies of sufficient size to permit the use of correlation and partial correlation techniques can therefore be anticipated.

Summary

Technique and equipment for measuring the extensibility and resistance to extension of gluten are described. A ring of gluten (internal and external diameter, 35 and 50 mm; weight, about 3 g) is stamped from a pressed disc, 3 mm thick, made manageable by freezing. After thawing in water at 25°C for 30 minutes, the ring is folded to form four strands, suspended from a balance, and stretched downwards by a pin moving at 40 cm per minute. Extensibility of the gluten is measured in centimeters by the distance it stretches before breaking, and resistance is recorded in grams by the balance. The machine is called a Stretchometer. Data showing the effects of changing the conditions for various steps of the testing procedure are reported.

When gluten is washed from a dough its resistance increases and its extensibility decreases with the increasing protein content of the gluten brought about by continued washing. The relation between

resistance and protein content is linear up to about 85% protein where additional washing continues to increase the resistance but removes little more nonprotein matter. The change in gluten properties during washing may result not only by purification but also from the work done on the glutens. The change in extensibility is not as marked as that in resistance, and the relation between extensibility and final protein content appears to be linear throughout.

Glutens were prepared from eight samples of Thatcher wheat flour ranging in protein content from 10.5 to 16.5%. Minimum and maximum mean values for the glutens from different flours were 40 and 85 g for resistance, and 28 and 36 cm for extensibility, and differences between flours were highly significant. Resistance and extensibility were inversely correlated ($r = -.95$; $1\% = .83$), but neither was correlated with the protein content or loaf volume of the flour. A differential behavior of glutens from different samples to washing was established; regressions for resistance (but not for extensibility) on gluten protein differed significantly.

Glutens from composite samples of six varieties showed no significant differences in extensibility, but mean resistance values ranged from 60 to 88 g and differed significantly. No significant relation was found between gluten properties and protein content or loaf volume of the flours. It was noted that the varieties were placed in the same order for decreasing resistance as for decreasing general strength of doughs as measured by the farinograph and the mixograph.

Acknowledgment

The authors are indebted to Mr. H. E. Rasmussen for his help in the construction of the Stretchometer.

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ESSENTIAL AMINO ACID COMPOSITION OF FEED MATERIALS

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A large number of feed materials have been analyzed for 12 amino acids by microbiological assay. Three microorganisms, *Lactobacillus arabinosus*, *Lactobacillus casei*, and *Streptococcus faecalis* R, have been employed in these determinations. *L. arabinosus* and *L. casei* have been used extensively and to a high degree of satisfaction in microbiological amino acid determinations. Assays using *S. faecalis* R were previously reported, but as details were not given they shall be discussed in this report.

An improvement of our earlier procedure for *S. faecalis* R was introduced by using acid production instead of turbidity as measurement of growth. The medium of Teply and Elvehjem (1945) for folic acid with *S. faecalis* R was modified for amino acid determinations. Sodium citrate was added to our *S. faecalis* R medium as an additional buffer. Higher acidity was produced with such a medium, which allowed evaluation of the assay by titration of the acid formed, but sodium citrate intensified caramelization of the glucose during autoclaving and consequently the detection of the indicator end point was hindered. This difficulty was overcome by sterilizing the medium separately by passing it through a Seitz filter. The medium was inoculated and added aseptically to the autoclaved culture tubes containing the hydrolyzates. This adaptation was found satisfactory in assays with *S. faecalis* R, provided pyridoxamine was added, except in assays for lysine and tyrosine. Their outstanding behavior will be referred to below.

The techniques establishing the reliability of the assays and their application to feed materials are described. The analyzed materials are appraised in terms of essential amino acids and their nutritional significance has been discussed.

Experimental Technique

Microbiological Procedure. Three microorganisms, *Lactobacillus arabinosus*, *Lactobacillus casei*, and *Streptococcus faecalis* R (formerly known as *Streptococcus lactis* R), were employed. Stock cultures were carried in neutral tomato juice agar, containing 400 ml clarified tomato

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TABLE I

COMPOSITION OF MEDIUM [1] FOR MICROBIOLOGICAL ASSAY OF AMINO ACIDS [2] WITH *Streptococcus faecalis* R

Components	Medium I [3] For turbidity measurement (medium autoclaved)	Medium II For titration measurement (medium autoclaved)	Medium III For titration measurement (medium sterilized by filtration)
Glucose	100.0 mg	100.0 mg	100.0 mg
Sodium acetate anhyd.	60.0 mg	60.0 mg	60.0 mg
Sodium citrate anhyd.	—	200.0 mg	200.0 mg
Adenine	0.5 mg	0.5 mg	0.5 mg
Guanine	0.5 mg	0.5 mg	0.5 mg
Uracil	0.5 mg	0.5 mg	0.5 mg
Xanthine	0.5 mg	0.5 mg	0.5 mg
K_2HPO_4	5.0 mg	5.0 mg	5.0 mg
KH_2PO_4	5.0 mg	5.0 mg	5.0 mg
$MgSO_4 \cdot 7\ H_2O$	2.0 mg	2.0 mg	2.0 mg
$MNSO_4 \cdot 4\ H_2O$	0.1 mg	0.1 mg	0.1 mg
NaCl	0.1 mg	0.1 mg	0.1 mg
$FeSO_4 \cdot 7\ H_2O$	0.1 mg	0.1 mg	0.1 mg
Thiamine hydrochloride	10.0 γ	10.0 γ	10.0 γ
Pyridoxine hydrochloride	2.0 γ	2.0 γ	—
Calcium pantothenate	2.0 γ	2.0 γ	2.0 γ
Riboflavin	2.0 γ	2.0 γ	2.0 γ
Nicotinic acid	4.0 γ	4.0 γ	4.0 γ
p-Aminobenzoic acid	2.0 γ	2.0 γ	2.0 γ
Biotin	0.05 γ	0.05 γ	0.05 γ
Pyridoxamine hydrochloride			10.0 γ
Folic acid [4]	2.5 mg units	2.5 mg units	2.5 mg units
dl Alanine		4.0 mg	
l(+) Arginine hydrochloride		2.0 mg	
Asparagine		4.0 mg	
l(−) Cystine		2.0 mg	
l(+) Glutamic acid		4.0 mg	
Glycine		2.0 mg	
l(−) Histidine hydrochloride		2.0 mg	
l(−) Hydroxyproline		2.0 mg	
dl Isoleucine		2.0 mg	
l(−) Leucine		2.0 mg	
dl Lysine hydrochloride		2.0 mg	
dl Methionine		2.0 mg	
dl Norleucine		2.0 mg	
dl Phenylalanine		2.0 mg	
l(−) Proline		2.0 mg	
dl Serine		2.0 mg	
dl Threonine		2.0 mg	
l(−) Tryptophane		2.0 mg	
l(−) Tyrosine		2.0 mg	
dl Valine		2.0 mg	

[1] The medium was adjusted to pH 6.8–7.0 and diluted to contain the contents of one assay tube in 5 ml of solution.

[2] The amino acid to be assayed was omitted from the medium.

[3] The medium in column I was used in assays with *L. arabinosus* and *L. casei*.

[4] Vitamin B_6 was employed in assays for tryptophane as the impure liver eluate contains some tryptophane. Norit-treated casein hydrolyzate was used in these assays (Greene and Black, 1944). One mg unit of folic acid is defined as the activity of 1 mg of a standard preparation of solubilized liver powder (Wilson & Co.). University of Texas publication No. 4137, p. 36, "Assay Method for Folic Acid," Mitchell and Snell.

juice, 10 g yeast extract, 10 g glucose, 5 ml each of salt A and B (Williams, 1941), and 15 g agar in one liter. The inoculum was prepared in tomato juice broth of pH 5.5 containing 400 ml tomato juice, 10 g Neopeptone, 10 g bactopeptonized milk, 10 g yeast extract, 10 g glucose, 5 ml each of salt A and B, and 2 ml of liver eluate prepared according to Hutchings, Bohonos, and Peterson (1941) in one liter of solution and incubated at 37°C for 18 hours before use.

The assay technique for *L. arabinosus* and *L. casei* has been described (Baumgarten, Garey, Olsen, Stone, and Boruff, 1944; and Baumgarten, Stone, and Boruff, 1945).

S. faecalis R has been employed (see Snell and Guirard, 1943, for preliminary report on its amino acid requirements) in microbiological assays of grain alcohol fermentation by-products (Baumgarten, Stone, and Boruff, 1945). In those earlier assays growth was measured by turbidity. The composition of the media for *S. faecalis* R is given in Table I. The medium in column I (it should be noted that this is the medium employed in assays with *L. arabinosus* and *L. casei*) was adequate in assays in which turbidity was considered a satisfactory measurement. The addition of 2% sodium citrate (medium, column II) buffers the medium sufficiently to allow production of the maximal amount of lactic acid. The dipotassium phosphate of Teply and Elvehjem's folic acid medium was omitted as no advantage was observed, and instead 0.6% sodium acetate was added. The addition of sodium citrate intensified the caramelization and dark color of the final bacterial suspension, and impeded titration against a color indicator.

This difficulty was overcome by sterilizing the medium and the samples separately. Suitable aliquots of the unknown neutral hydrolyzates or the neutralized solutions of the amino acid for establishing the standard curve were introduced into the assay tubes (25 × 200 mm) and the volume adjusted to 5 ml with distilled water. The tubes were plugged with cotton and autoclaved. The medium was sterilized separately by passing through a Seitz filter and then inoculated with the bacterial suspension. Five ml of the inoculated medium were added aseptically to the autoclaved tubes containing the amino acid solutions or the hydrolyzates. It was observed, however, that acid production was only 80% with such a medium as compared to the acid formed with the same medium sterilized by autoclaving. It was established that this was due to a pyridoxamine deficiency and that the medium (column III) containing pyridoxamine supported maximal growth and gave identical results regardless of the method of sterilization. An explanation of this phenomenon was indicated by Snell and Rannefeld (1945) who showed that pyridoxamine is the active vitamin

for *S. faecalis* R and not pyridoxine itself. Snell (1945a) proved that pyridoxamine was formed during heating of pyridoxine with amino acid solutions, and established (1945) the natural occurrence of pyridoxamine. From our experiments it is apparent that the addition of

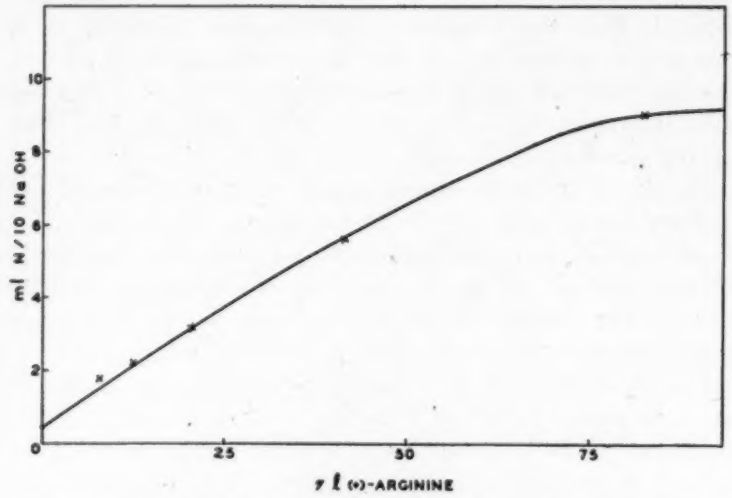

Fig. 1. Standard curve for arginine with *S. faecalis* R, employing medium III.

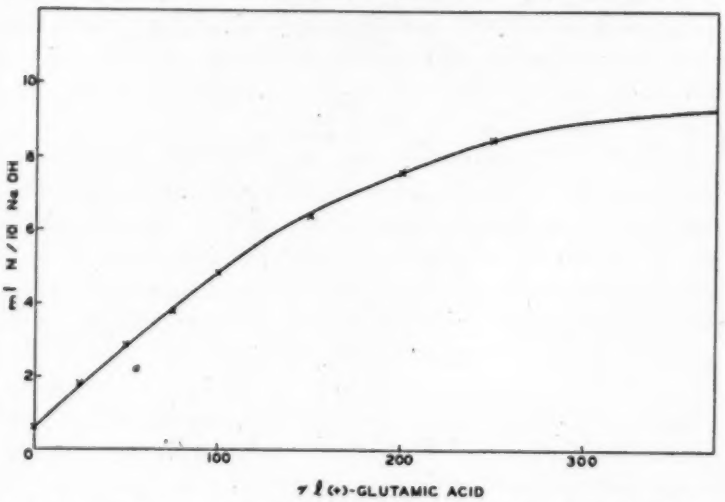

Fig. 2. Standard curve for glutamic acid with *S. faecalis* R, employing medium III.

pyridoxamine was essential if the medium was not heat sterilized. It must be assumed that suboptimal amounts of pyridoxamine were formed at 30°C by the interaction of pyridoxine and amino acid solutions. Assay results tended to be higher when the filter-sterilized

medium devoid of pyridoxamine was employed. More titratable acid was formed in the tubes containing the natural hydrolyzates than in those tubes used to establish the standard curve when compared in terms of assayed amino acid present. This was due to the fact that

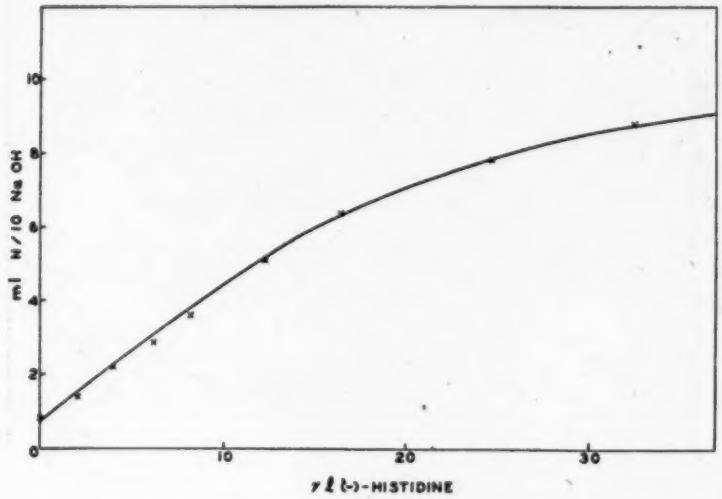

Fig. 3. Standard curve for histidine with *S. faecalis* R, employing medium III.

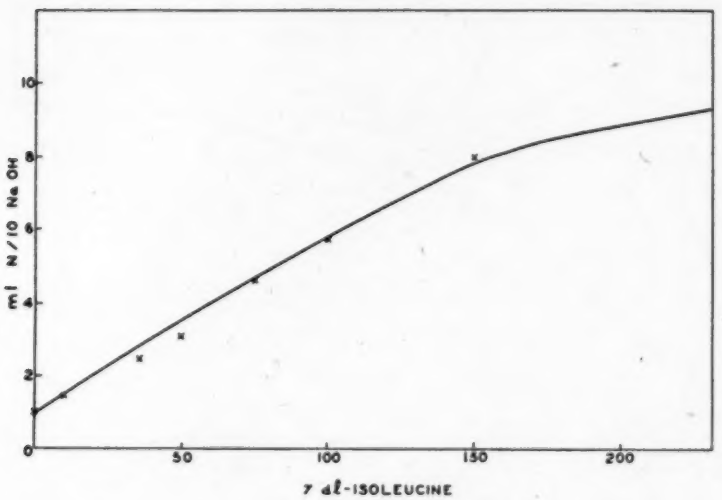

Fig. 4. Standard curve for isoleucine with *S. faecalis* R, employing medium III.

pyridoxamine was present in the hydrolyzates and led to a wrong interpretation of the results. Again, the necessity for nutritionally complete media must be pointed out. The role of glutamic acid (Snell, 1945a) in the transamination reaction was strongly indicated

as its assays showed the greatest discrepancies in the absence of pyridoxamine.

The medium in column III (Table I) was adopted for assays of arginine, glutamic acid, histidine, isoleucine, leucine, methionine,

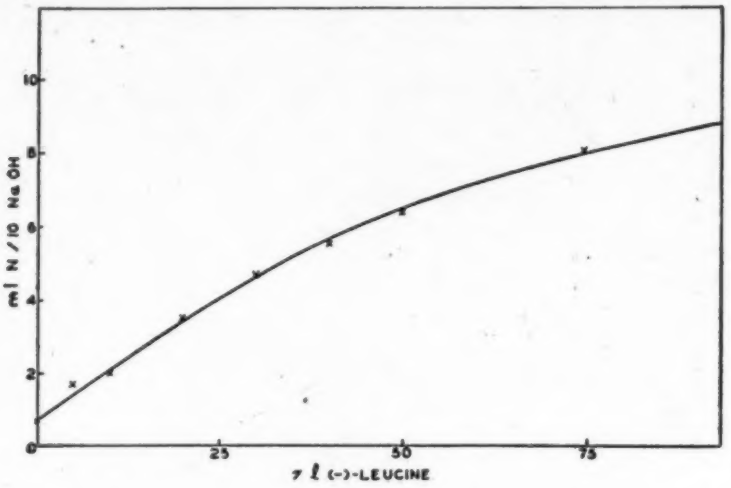

Fig. 5. Standard curve for leucine with *S. faecalis* R, employing medium III.

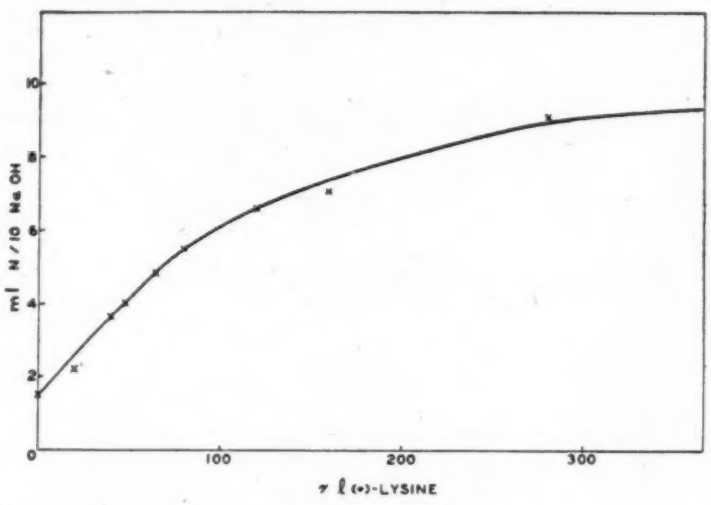

Fig. 6. Standard curve for lysine with *S. faecalis* R, employing medium II.

serine, threonine, tryptophane, and valine with *S. faecalis* R. Standard curves for these amino acids are presented in Figures 1 to 5, 7 to 10, and 12. Lysine (Figure 6) and tyrosine (Figure 11) were determined employing the medium in column II.

The essential character of tyrosine was dependent on the amount of pyridoxamine present in the medium. The amount of pyridoxamine formed during autoclaving of the pyridoxine with the amino acid solutions or hydrolyzates did not interfere; however, the addition of

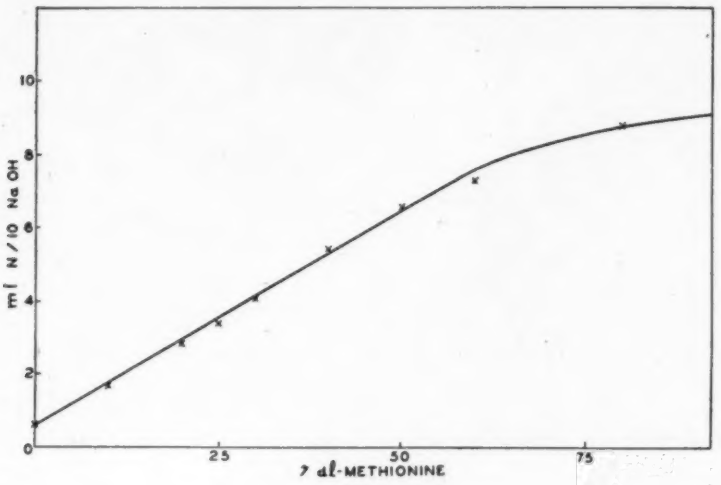

Fig. 7. Standard curve for methionine with *S. faecalis* R, employing medium III.

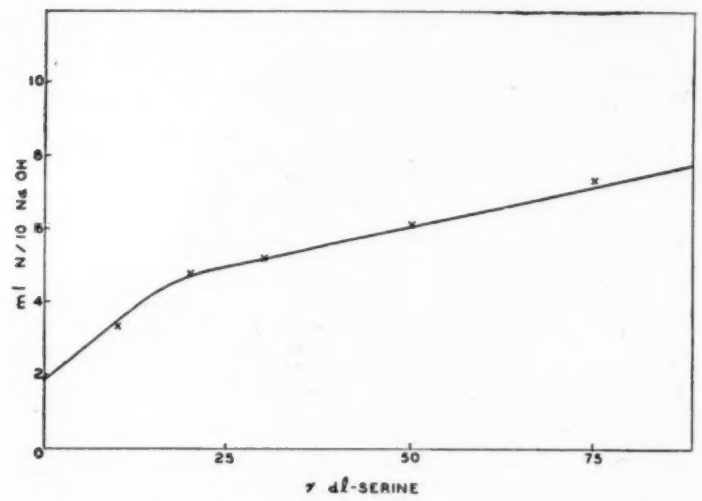

Fig. 8. Standard curve for serine with *S. faecalis* R, employing medium III.

1 μg of pyridoxamine per assay tube resulted in near maximal blank titration. Results obtained with the medium given in column II were satisfactory and in close agreement with values determined with *L. casei*. Experiments showed that pyridoxal, the pyridoxine analogue

essential for the growth of *L. casei*, did not interfere with tyrosine determinations employing *L. casei*.

Lysine exhibited an unexplained behavior. Standard curves of

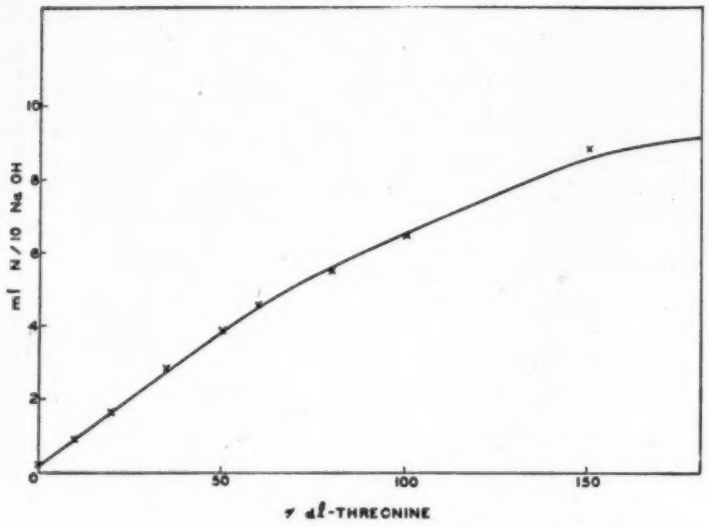

Fig. 9. Standard curve for threonine with *S. faecalis* R, employing medium III.

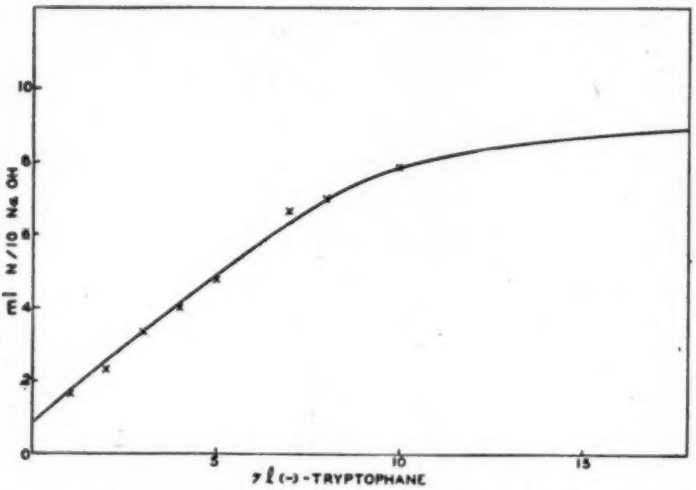

Fig. 10. Standard curve for tryptophane with *S. faecalis* R, employing medium III.

lysine with *S. faecalis* R established with medium III showed a higher blank titration than the next tubes, resulting in a dip in the curve.

The medium in column II was found satisfactory for both lysine and tyrosine and was adopted for their determinations.

Reliability of Assays with S. faecalis R. Smooth standard curves, recovery of added amino acid within the limit of experimental error, and identical results at various levels of concentration of amino acid

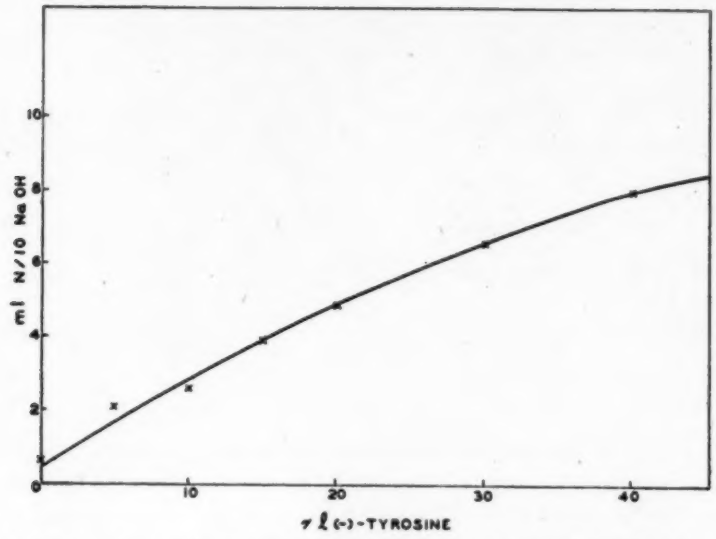

Fig. 11. Standard curve for tyrosine with *S. faecalis* R, employing medium II.

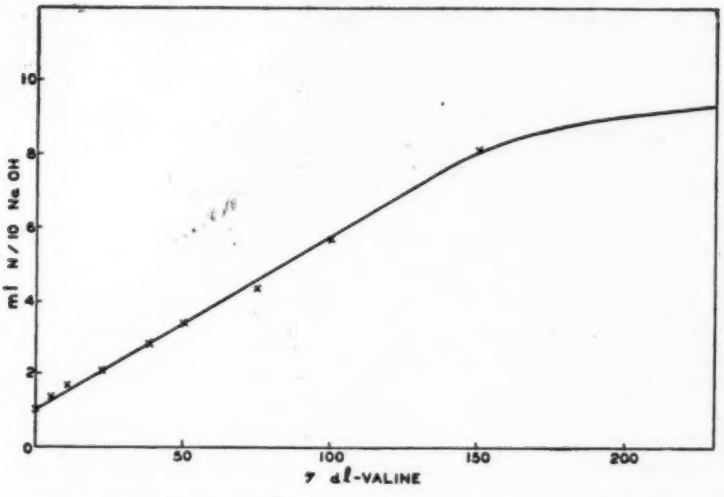

Fig. 12. Standard curve for valine with *S. faecalis* R, employing medium III.

were established in assays of amino acid with *S. faecalis* R. Recovery figures for methionine and serine are included in this report as they were not reported previously. These recovery experiments were carried out by addition of amino acid to the material. From these

results it can be observed that recovery is well within the limits of the experimental error, a fact which invalidates claims that growth inhibitors might be present in such hydrolyzates (Table II).

TABLE II

RECOVERY DATA ON METHIONINE AND SERINE IN ASSAYS WITH *S. faecalis* R

Material investigated	Per cent found		Per cent recovery
	Methionine	Serine	
Corn	0.15	—	—
Corn + 0.25% *l*(−) methionine	0.38	—	92
Wheat	0.15	0.56	—
Wheat + 0.25% *l*(−) methionine	0.38	—	92
Wheat + 1.25% *l*(−) serine	—	1.7	91
Corn solubles	—	1.2	—
Corn solubles + 1.25% *l*(−) serine	—	2.5	104
Tankage	—	2.2	—
Tankage + 1.25% *l*(−) serine	—	3.4	96

In addition two commercial purified proteins were analyzed with five microorganisms for the purpose of comparing the results and proving their reliability. Twelve amino acids were determined in casein and gelatin and the results compiled in Tables III and IV. In most instances the values agreed very closely. The results for glutamic acid were discussed previously (Baumgarten, Mather, and Stone,

TABLE III

COMPARISON OF AMINO ACID COMPOSITION OF CASEIN [1] AS DETERMINED MICROBIOLOGICALLY WITH FIVE MICROORGANISMS

	Optical form of amino acid used for standard curve	Amino acid composition (dry matter basis) as determined by [2]					Chemical [3] analysis
		L. acidophilus	*L. arabinosus*	*L. casei*	*L. mesenteroides*	*S. faecalis* R	
		%	%	%	%	%	%
Arginine	*l*(+)	4.1	—	3.6	4.0	3.8	3.8
Histidine	*l*(−)	3.7	—	—	3.2	3.3	2.3
Isoleucine	*dl*	7.3	7.7	7.8	7.6	7.5	6.0
Leucine	*l*(−)	10.8	10.7	11.2	11.0	9.6	11.3
Lysine	*l*(+)	—	—	—	—	8.3	6.4
Methionine	*dl*	3.0	2.7	—	3.1	3.0	3.2
Phenylalanine	*dl*	4.9	—	5.0	—	—	4.9
Serine	*dl*	—	—	—	—	6.3	6.0
Threonine	*dl*	—	4.5	—	—	4.1	3.6
Tryptophane	*l*(−)	0.9	1.0	0.9	—	0.9	1.6
Tyrosine	*l*(−)	5.0	—	5.5	—	5.0	5.9
Valine	*dl*	—	7.4	6.9	8.1	7.5	6.5

[1] Labco casein: 3.7% moisture, 14.84% nitrogen (on dry basis).
[2] For description of organisms and medium employed see Baumgarten, Mather, and Stone (1945).
[3] Block and Bolling (1945).

TABLE IV

COMPARISON OF AMINO ACID COMPOSITION OF GELATIN [1] AS DETERMINED MICROBIOLOGICALLY WITH FIVE MICROORGANISMS

	Optical form of amino acid used for standard curve	Amino acid composition (dry matter basis) as determined by [2]					Chemical [3] analysis
		L. acidophilus	*L. arabinosus*	*L. casei*	*L. mesenteroides*	*S. faecalis* R	
		%	%	%	%	%	%
Arginine	*l*(+)	8.2	—	7.9	8.4	8.3	8.2
Histidine	*l*(−)	1.0	—	0.8	0.8	1.0	1.1
Isoleucine	*dl*	1.9	1.9	2.3	1.9	1.9	1.8
Leucine	*l*(−)	3.6	3.7	3.6	3.6	3.2	4.0
Lysine	*l*(+)	—	—	—	—	5.1	4.7
Methionine	*dl*	1.0	0.9	—	0.9	1.0	0.9
Phenylalanine	*dl*	2.3	—	2.1	—	—	2.2
Serine	*dl*	—	—	—	—	4.0	3.6
Threonine	*dl*	—	2.0	—	—	1.9	1.6
Tyrosine	*l*(−)	0.8	—	0.7	—	0.6	0.2
Valine	*dl*	—	3.1	2.5	3.7	2.7	2.7

[1] Difco gelatin: 10.8% moisture, 17.23% nitrogen (dry basis).
[2] For description of organisms and medium employed see Baumgarten, Mather, and Stone (1945).
[3] Block and Bolling (1945).

TABLE V

COMPARISON OF AMINO ACID VALUES EMPLOYING THE DIFFERENT MEDIA [1] IN ASSAYS WITH *S. faecalis* R

Amino acid	Medium employed	Corn	Wheat	Corn solubles	Wheat-Milo grains with solubles	Milo grains with solubles
Arginine	I	—	—	—	1.0	0.78
	III	—	—	—	1.0	0.80
Histidine	I	—	0.39	—	0.64	0.76
	II	—	—	—	0.61	0.62
	III	—	0.28	—	0.58	0.65
Glutamic acid	I	2.0	3.9	—	—	—
	III	1.96	3.6	—	—	—
Lysine	I	0.27	0.38	—	—	—
	II	0.28	0.38	—	—	—
Serine	II	—	0.56	1.15	—	—
	III	—	0.64	1.22	—	—
Threonine	I	—	—	—	0.96	1.0
	III	—	—	—	0.89	0.96
Tyrosine	I	—	0.12	—	—	0.75
	II	—	0.10	—	—	0.77

[1] The media are described in Table I, columns I, II, and III.

1945). For comparison, values determined chemically were included (Block and Bolling, 1945).

Amino acid values determined with *S. faecalis* R were independent regardless of the medium (Table I) employed. Comparisons were carried out for several amino acids employing the different media and the results are given in Table V. For isoleucine, leucine, methionine, tryptophane, and valine, results obtained with medium III with *S. faecalis* R were compared with those determined with *L. arabinosus*. These data are given in Table VI. These compilations might appear

TABLE VI

COMPARISON OF ASSAY RESULTS OF SOME NATURAL PRODUCTS DETERMINED WITH *S. faecalis* R AND *L. arabinosus* [1]

Amino acid	Determined with	Corn	Wheat	Fish solubles	Red Dog flour	Milo-wheat solubles	Milo-wheat grains with solubles
Isoleucine	*S. faecalis* R	0.36	0.50	—	—	—	—
	L. arabinosus	0.44	0.57	—	—	—	—
Leucine	*S. faecalis* R	1.16	0.86	—	—	—	—
	L. arabinosus	1.37	0.91	—	—	—	—
Methionine	*S. faecalis* R	0.19	0.17	—	—	0.40	—
	L. arabinosus	0.18	0.14	—	—	0.36	—
Tryptophane	*S. faecalis* R	—	—	0.30	0.17	—	—
	L. arabinosus	—	—	0.30	0.16	—	—
Valine	*S. faecalis* R	0.40	—	—	—	—	1.5
	L. arabinosus	0.46	—	—	—	—	1.7

[1] Medium III was employed with *S. faecalis* R; medium I with *L. arabinosus*.

superfluous, but similar results obtained on natural hydrolyzates strengthen the degree of reliability which can be assigned to such results, and they give additional information with respect to the possible occurrence of growth inhibitors.

Specificity of Response. On first assumption only the naturally occurring *l* amino acids were supposed to possess growth-promoting activity (Stokes and Gunness, 1944). The substitution of optical antipodes or closely related compounds of specific amino acids for *L. arabinosus*, *L. casei*, and *S. faecalis* R has been investigated. Only in the case of glutamic acid (Baumgarten, Mather, and Stone, 1945) has the *d* form been found to be partially active. Under our experimental conditions *dl* leucine possessed half the activity of *l*(−) leucine and *d*(+) leucine was entirely ineffective to promote growth of any of the three organisms (Table VII). Hegsted (1945) reported *d*(+) leucine

TABLE VII

RESPONSE OF *L. arabinosus*, *L. casei*, AND *S. faecalis* R TO THE DIFFERENT OPTICAL FORMS OF LEUCINE

Leucine added per tube of 10 ml			Growth produced by [1]		
l(−)	*dl*	*d*(+)	*L. arabinosus*	*L. casei*	*S. faecalis* R
γ	γ	γ	ml *N*/10 NaOH	ml *N*/10 NaOH	ml *N*/10 NaOH
0	0	0	0.9	0.8	0.6
25	0	0	4.15	3.9	3.7
0	50	0	4.0	3.75	3.5
50	0	0	6.55	6.4	6.05
0	100	0	6.45	6.5	5.7
0	0	100	0.9	0.9	0.7
50	0	21,000	—	—	7.15
100	0	0	8.5	8.1	—
100	0	21,000	9.0	8.8	—

[1] Medium I was employed with *L. arabinosus* and *L. casei*; medium III with *S. faecalis* R. The media were complete, only leucine was omitted.

TABLE VIII

RESPONSE OF THE MICROORGANISMS TO THE *l* AND *dl* FORMS OF LYSINE, METHIONINE, TRYPTOPHANE, AND VALINE

Medium contained per tube		Growth produced by [1]		
l	*dl*	*L. arabinosus*	*L. casei*	*S. faecalis* R
γ	γ	ml *N*/10 NaOH	ml *N*/10 NaOH	ml *N*/10 NaOH
Lysine				
0	0	—	—	1.6
40	0	—	—	3.0
0	80	—	—	2.9
100	0	—	—	4.65
0	200	—	—	4.55
Methionine				
0	0	—	—	0.65
10	0	—	—	2.7
0	20	—	—	3.0
35	0	—	—	7.6
0	70	—	—	8.1
Tryptophane				
0	0	1.9	—	0.0
1	0	3.0	—	1.65
0	2	3.15	—	1.7
2	0	4.15	—	2.3
0	4	4.3	—	2.5
3	0	4.8	—	3.35
0	6	5.1	—	3.5
Valine				
0	0	0.8	1.3	82.2
10	0	2.45	2.8	63.75
0	20	2.55	2.7	61.5
20	0	3.7	3.6	56
0	40	3.9	3.6	53
50	0	7.0	5.55	35
0	100	7.2	5.3	34

[1] Medium I was used with all three organisms, in the case of valine. In the determinations of lysine, methionine, and tryptophane, medium III was employed with *S. faecalis* R. The figures with *S. faecalis* R for valine are turbidity readings on a arbitrary scale.

active for *L. arabinosus*. Neither was an inhibition of growth observed when *d*(+) leucine was added to assay tubes containing *l*(−) leucine under our experimental conditions, contrary to reports of Fox, Fling, and Bollenback (1944) for *L. arabinosus*.

S. faecalis R required twice the amount of *dl* lysine to produce the same quantity of acid produced from *l*(+) lysine. Similar results were observed with methionine and tryptophane for *L. arabinosus* and *S. faecalis* R and with valine for *L. arabinosus*, *L. casei*, and *S. faecalis* R (Table VIII).

A number of compounds derived from the corresponding amino acids were tested for growth-promoting activity (Table IX). His-

TABLE IX

RESPONSE OF *L. arabinosus* AND *S. faecalis* R TO COMPOUNDS RELATED TO GROWTH-PROMOTING AMINO ACIDS IN MEDIA DEVOID OF THE AMINO ACID

Medium contained	Growth produced by [1]	
	L. arabinosus ml *N*/10 NaOH	*S. faecalis* R ml *N*/10 NaOH
0 *l*(−) histidine	—	0.8
0 *l*(−) histidine + 500 γ histamine	—	1.0
160 γ *l*(−) histidine	—	9.2
160 γ *l*(−) histidine + 2000 γ histamine	—	9.2
0 *l*(+) lysine	—	1.5
0 *l*(+) lysine + 500 γ cadaverine	—	2.3
80 γ *l*(+) lysine	—	5.5
80 γ *l*(+) lysine + 4000 γ cadaverine	—	5.4
0 *l*(−) tryptophane	0.65	0.0
0 *l*(−) tryptophane + 100 γ indole	6.8	0.0
0 *l*(−) tryptophane + 100 γ anthranilic acid	5.4	0.0
0 *l*(−) tryptophane + 500 γ tryptamine	1.4	0.0
0 *l*(−) tryptophane + 1000 γ indole-3-acetic acid	1.8	0.0
10 γ *l*(−) tryptophane	6.6	5.3
10 γ *l*(−) tryptophane + 4000 γ indole	0.65	0.0
10 γ *l*(−) tryptophane + 4000 γ anthranilic acid	6.6	4.3
10 γ *l*(−) tryptophane + 4000 γ tryptamine	6.3	4.2
10 γ *l*(−) tryptophane + 4000 γ indole-3-acetic acid	6.3	5.7

[1] Medium I was employed with *L. arabinosus;* medium III with *S. faecalis* R.

tamine did not replace histidine, nor did cadaverine replace lysine for *S. faecalis* R. Indole and anthranilic acid were reported to be stimulatory for *L. arabinosus* (Greene and Black, 1943). Employing the present experimental technique, indole replaced tryptophane for *L. arabinosus*, but was entirely inactive in replacing tryptophane for *S. faecalis* R. In high concentrations it inhibited growth for both organisms. Anthranilic acid substituted for tryptophane only for *L. arabinosus* but not for *S. faecalis* R. No inhibitory activity was exhibited. Tryptamine and indole-3-acetic acid were ineffective.

Recently Stokes and Gunness (1945) demonstrated that only the *l* form of serine promoted growth for *L. delbruckii.*

Preparation and Assay of Samples. Preparation of the samples for tryptophane determination was accomplished by autoclaving with saturated barium hydroxide solution for 7 hours at 15 pounds pressure (Greene and Black, 1944). Refluxing for 20 hours with 20% hydrochloric acid was employed in processing the materials for the assay of the other amino acids. Stability of the individual amino acids under the conditions of hydrolysis was investigated by adding the amino acids one by one to some samples and determining the percentage of recovery (see Baumgarten, Stone, and Boruff, 1945).

Arginine, phenylalanine, and tyrosine were determined with *L. casei;* isoleucine, leucine, methionine, tryptophane, and valine with *L. arabinosus;* and arginine, histidine, isoleucine, leucine, lysine, methionine, serine, threonine, tryptophane, tyrosine, and valine with *S. faecalis* R. The glutamic acid values were included for comparison and were discussed previously (Baumgarten, Mather, and Stone, 1945).

The tryptophane determinations were carried out using norit-treated casein hydrolyzate supplemented with cystine as the source of amino acids (Greene and Black, 1944). In these assays crystalline vitamin B_c was substituted for liver eluate as the latter nearly always contained some tryptophane. In the calculation of the tryptophane content of the samples, the observed values were multiplied by two since the tryptophane is completely racemized by alkaline hydrolysis.

Discussion of Results

The analytical data are compiled in Table X. The results are expressed in the percentage of amino acid contained in the dried materials. The nitrogen content of the samples is given, and the nitrogen percentage accounted for by the amino acids has been calculated.

Relatively little data are available in the literature on the amino acid composition of complex materials such as the feeds investigated. A very excellent review covering the literature up to 1943 has been compiled by Block and Bolling (1945). We shall not attempt to compare individual values, as samples of this nature vary considerably, depending on their source and origin. It might be sufficient to note that for the materials contained in Block and Bolling's compilation the values given are of the same order of magnitude as ours, with the exception of leucine and isoleucine. The leucine values are higher and the isoleucine values lower than our results, which might be attributed to the lack of dependability of the chemical method for the two amino acids.

TABLE X

AMINO ACID COMPOSITION OF FEEDS
(Dry matter basis)

	Nitrogen	Arginine	Glutamic acid [1]	Histidine	Isoleucine	Leucine	Lysine	Methionine	Phenylalanine	Serine	Threonine	Tryptophane	Tyrosine	Valine	Per cent nitrogen accounted for by amino acids in dry material
Grains & grain by-products	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Wheat	2.6	0.5	3.9	0.4	0.7	1.0	0.4	0.2	0.7	0.7	0.4	0.2	0.1	0.7	47.28
Granular wheat flour	2.4	0.4	3.8	0.3	0.7	1.0	0.3	0.1	0.7	0.6	0.3	0.1	0.2	0.6	46.01
Corn	1.5	0.4	2.1	0.3	0.6	1.4	0.3	0.2	0.5	0.8	0.3	0.05	0.2	0.5	62.66
Rye	2.9	0.6	3.6	0.4	0.7	1.0	0.6	0.1	0.7	0.7	0.4	0.1	0.2	0.7	42.48
Milo maize	1.9	0.4	2.6	0.3	0.7	1.8	0.3	0.1	0.6	0.6	0.3	0.1	0.2	0.7	44.35
Ground barley	2.9	0.5	3.2	0.3	0.7	1.0	0.4	0.2	0.7	0.5	0.4	0.2	0.2	0.7	39.59
Barley malt	2.4	0.5	2.5	0.3	0.7	0.9	0.6	0.2	0.6	0.5	0.4	0.2	0.2	0.7	45.58
Gluten feed	4.6	0.7	4.5	0.8	1.3	3.0	0.8	0.4	1.0	0.9	1.0	0.1	0.4	1.5	44.41
Gluten meal	7.1	1.2	9.4	1.0	2.4	7.3	0.8	0.9	2.5	1.7	1.4	0.2	1.1	2.1	52.76 [2]
Wheat bran	3.0	0.9	—	0.5	0.8	1.1	0.6	0.2	0.6	1.0	0.4	0.3	0.2	0.9	37.88
Oats, finely ground	2.7	0.7	2.9	0.4	0.7	1.1	0.6	0.2	0.8	0.4	0.4	0.1	0.2	0.7	45.36
Standard wheat middlings	3.7	1.3	4.5	0.6	1.1	1.3	0.6	0.2	1.0	0.9	0.6	0.2	0.3	1.2	51.16
Red Dog flour	3.8	0.9	—	0.6	1.0	1.3	0.7	0.2	0.5	1.1	0.6	0.2	0.2	1.2	34.76 [2]
Distillers' by-products															
Corn grains with solubles	5.1	0.9	5.7	0.8	2.0	2.4	0.7	0.5	2.1	1.9	1.1	0.1	0.7	1.8	49.77
Corn solubles	4.4	0.6	4.3	0.7	1.5	1.4	0.7	0.4	1.7	1.2	0.9	0.1	0.6	1.4	43.80
Rye grain with solubles	5.1	1.2	5.5	0.7	1.6	2.2	1.1	0.4	1.4	1.3	1.1	0.3	0.5	1.7	47.16
Rye solubles	6.4	1.4	—	0.8	2.0	1.9	0.7	0.5	1.8	1.7	1.2	0.2	0.6	2.1	33.18 [2]
Wheat grains with solubles	5.4	1.4	9.3	0.8	2.0	2.6	1.0	0.5	1.7	1.8	1.2	0.3	0.6	2.1	57.08
Milo grains with solubles	5.8	1.1	7.6	1.0	0.9	1.8	0.9	0.5	1.9	2.2	1.1	0.3	0.9	2.1	48.10
Wheat-Milo grains with solubles	5.1	1.2	7.7	0.8	1.8	3.5	1.0	0.5	1.8	1.7	1.0	0.3	0.8	1.9	57.96
Wheat-Milo solubles	4.9	1.2	7.9	0.7	1.6	1.9	0.8	0.4	1.4	2.1	1.1	0.2	0.6	1.5	53.78
Butyl alcohol fermentation by-products	4.9	0.7	—	0.5	1.8	2.2	1.2	0.4	1.0	1.0	0.5	0.2	0.5	1.7	32.59 [2]

TABLE X—*Continued*

TABLE X—*Continued*

	Nitrogen	Arginine	Glutamic acid [1]	Histidine	Isoleucine	Leucine	Lysine	Methionine	Phenylalanine	Serine	Threonine	Tryptophane	Tyrosine	Valine	Per cent nitrogen accounted for by amino acids in dry material
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Vegetable protein concentrates															
Cottonseed meal	6.4	3.0	6.8	1.1	1.8	2.2	1.5	0.5	2.2	1.8	1.1	0.4	0.6	1.8	53.22
Extracted soybean meal	9.1	3.0	9.0	1.7	3.5	4.5	3.0	0.9	2.8	2.4	2.1	0.6	1.2	3.0	56.00
Expelled soybean meal	7.6	2.8	7.8	1.2	2.9	4.0	3.2	0.4	2.2	2.2	2.2	0.5	0.9	2.6	59.63
Animal by-products															
Tankage	11.3	4.3	7.9	1.9	2.8	6.6	3.5	0.6	3.1	2.3	2.4	0.5	1.5	4.0	51.12
Meat scraps	10.0	3.4	6.4	2.2	2.1	5.0	3.8	0.7	3.2	2.3	2.2	0.4	1.4	3.8	52.28
Liver meal	13.0	4.0	8.1	1.8	3.7	6.7	4.9	1.2	3.9	2.7	2.6	0.5	1.8	4.2	48.85
Liver powder	10.0	1.7	8.3	1.1	1.3	1.8	4.2	0.4	0.7	2.2	1.5	0.2	0.6	1.2	34.85
Fish meal	12.0	4.5	9.2	2.3	4.9	6.4	6.6	1.8	3.5	2.2	3.1	0.6	2.3	4.5	60.96
Fish solubles	11.1	2.6	—	5.0	1.6	1.8	2.3	0.8	1.2	2.2	1.2	0.3	0.5	1.5	34.94 [2]
Casein	15.5	4.1	22.5	4.1	8.0	10.8	8.5	3.1	5.1	6.8	4.5	1.0	5.4	7.8	76.53
Miscellaneous feed supplements															
Animal feed yeast	8.3	2.0	5.4	1.4	3.4	3.4	3.3	1.5	2.6	4.2	2.4	0.6	1.0	2.9	55.38
Irradiated dried yeast	8.8	2.2	6.3	1.2	3.8	4.1	4.8	0.7	2.3	3.0	2.8	0.6	1.0	3.0	55.16
Dried buttermilk	6.2	1.2	7.0	1.0	2.9	3.7	2.6	0.8	1.6	1.5	1.7	0.5	1.1	3.0	57.56
Dried skimmilk	6.4	1.2	7.3	1.3	2.9	3.7	2.9	0.9	2.0	1.7	1.7	0.4	0.9	2.4	58.77
Dried whey	2.7	0.3	1.7	0.2	1.0	1.2	0.8	0.2	0.5	0.6	0.8	0.1	0.1	0.8	37.82
Dried cheese whey	2.5	0.2	1.9	0.2	1.0	1.2	0.9	0.1	0.3	0.4	1.9	0.1	0.1	0.7	45.01
Dehydrated alfalfa	3.1	0.6	1.4	0.4	1.0	1.2	0.7	0.2	0.9	0.6	0.7	0.2	0.3	0.8	37.85
Dehydrated grass	3.1	1.3	3.8	0.6	1.8	2.6	1.4	0.4	1.7	1.2	1.3	0.4	0.6	2.0	81.20
Grass juice extract	5.3	0.3	1.5	0.2	0.6	0.7	0.7	0.1	0.3	1.1	0.6	0.1	0.2	0.8	14.91
Water-soluble fraction of grass	2.7	0.1	3.2	0.2	0.2	0.3	0.3	0.1	0.1	0.6	0.3	0.1	0.2	0.3	24.21

[1] Glutamic acid values were determined with *S. faecalis* R using *l*(−) glutamic acid as the standard. For discussion of these values see Baumgarten, Mather, and Stone (1945).

[2] Nitrogen accounted for by 12 amino acids, as glutamic acid was not determined in this material.

The amino acid composition of tankage, meat scraps, and whole corn (Block and Bolling, 1943) and of yeast and casein (Block and Bolling, 1945a) has been investigated. In the earlier publication leucine and isoleucine were determined chemically, and in the second report the results were obtained by microbiological assay. Our results agreed well with the microbiological but not with the chemical values. The results on the other amino acids compared favorably.

Several procedures have been employed in the determination of tryptophane. Dried yeast was found to contain by chemical analysis 1.1% tryptophane (Kratzer, 1944; and Ryan and Brand, 1944) and 1.32% (Greene and Black, 1944) by microbiological assay, while our value was 1.15%. All results were calculated to 16% nitrogen for comparison. Our microbiological values agreed well with the other chemical tryptophane values of Kratzer.

Our microbiological assay values compared well with results obtained by Ryan and Brand (1944) for tyrosine and leucine; by Kuiken and co-workers (1943) for leucine, isoleucine, and valine; by Grau and Almquist (1943) for methionine.

Serine, although nonessential, has been included in this study because heretofore very few values for it have been reported.

Results obtained by microbiological assay compared favorably with recent chemical values. It has been stated (Stokes and Gunness, 1945; and Dunn *et al.*, 1944) that microbiological assays should be applied only to pure proteins and not to complex substances such as feed materials investigated by us, as some inhibiting or stimulatory substances possibly affecting the assay might be present. However, the possible effect of such interfering substances was eliminated to a large extent by assaying at different levels of concentration of the hydrolyzates. Different values should result at the various levels in an unsatisfactory assay, but this was not the experimental result. Recovery results are an additional safeguard to rule out such a possibility. It may be argued that biological methods are more specific than chemical methods, as biological systems in general respond to a specific nutrilite, whereas in colorimetric analysis of amino acids related compounds such as amines can produce a similar color that is impossible to differentiate. For instance, in tryptophane determination the color-producing compound is indole.

Undoubtedly both the chemical and biological methods are of value, but for comparative values on practical proteins the microbiological technique appears to be of greater worth. A large number of samples can be analyzed at one time and such comparative values are of great reliability.

Nutritional Significance of Results. The qualitative amino acid requirements for rats, chicks, and human beings have been established. Rose (1938) has shown 10 amino acids—arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane, and valine—indispensable for the rat. The chick requires in addition glycine (Hegsted, 1944; Almquist and Grau, 1944); the status of the glutamic acid is uncertain. Human beings require isoleucine, leucine, phenylalanine, lysine, threonine, and tryptophane (Rose, Haines, Johnson, and Warner, 1943), while arginine and histidine are dispensable (Rose, 1945).

Almquist (1945) reported quantitative requirements for the chick: 0.9% arginine, 0.8–1.0% glycine, 0.8–1.0% isoleucine, 0.15% histidine, 1.0–2.0% leucine, 0.9% lysine, 0.9% methionine, 1.0% phenylalanine, 0.5–1.0% threonine, 0.25% tryptophane, and 1.0% valine. It is well known that cereal grains are deficient in lysine and tryptophane. Distillers' by-products are considerably higher in these amino acids and constitute a good protein source. Protein concentrates as well as animal by-products are well balanced in this respect. The milk products contain fairly high proportions of lysine and tryptophane.

From such data it should be possible to evaluate a diet or ration in the light of the amino acid requirements. In addition, however, the digestibility of the proteins ingested should be known.

Summary

An improved medium for amino acid assays with *S. faecalis* R has been described. It allows evaluation of the results by the titration of the acid produced, as under our assay conditions maximal production of lactic acid and optimal utilization of the glucose was achieved. This assay technique is a simplification of our earlier procedure.

The specificity of response of the three microorganisms, *L. arabinosus*, *L. casei*, and *S. faecalis* R, to their essential amino acid and their possible replacement by metabolites or predecessors of these amino acids have been investigated.

A number of feed materials has been analyzed for 12 amino acids—arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophane, tyrosine, and valine. The results are discussed in view of the literature values and their nutritive significance is indicated.

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EFFECT OF METHOD OF COMBINING THE INGREDIENTS UPON THE QUALITY OF THE FINISHED CAKE [1]

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During the last few years, new methods of mixing cake ingredients have been introduced. These vary the order of combining the ingredients and are tending to replace the conventional creaming method which has long been followed. Several methods of mixing are now recommended for commercial bakeries; for home use the trend is towards the use of short mixing times.

Several investigators have emphasized the importance of the emulsion in the batter in relation to the quality of the finished cake. Nason (1939) pointed out that when the egg was added slowly a more stable emulsion was formed. Grewe (1937) had previously made the same observation. She had also found the emulsion to be more stable when the specific gravity was greater than 0.75. Halliday and Noble (1933) stated that when the emulsion was in a stable form the cakes produced were more velvety and would retain their freshness for a longer time than those made from a batter in which the emulsion broke.

Bailey and LeClerc (1935) observed that a water-in-fat emulsion resulted when eggs and milk were added gradually during the creaming process. When these were added too rapidly, a curdled mass was formed as the emulsion changed from a water-in-fat to a fat-in-water dispersion. Collins and Sunderlin (1940) found that batters of high viscosity were associated with a water-in-oil type of emulsion and those of low viscosity with an oil-in-water emulsion. Lowe (1943) stated that thin-pouring batters produced inferior cakes. She also

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observed that curdled batters, if the flecks were small, often produced desirable cakes.

Lowe and Nelson (1939) pointed out that the method of combining the ingredients affected the viscosity of the batter, and that the more viscous the batter the higher the score given the cake. They referred to work by Buel in which she found that if the batter were viscous enough to stand in waves when poured into the pan, a good cake could be expected. Pyke and Johnson (1940) made similar observations regarding the relationship between the viscosity of the batter and the quality of the finished cake. They concluded that a batter with a high specific gravity and low viscosity yielded a cake of small volume, coarse grain, and a somewhat harsh, rubbery texture which was not of the velvety type. Alexander (1931) found a direct relation between specific gravity of batter and quality of the finished cake.

A cake which has been mixed just the right amount, as described by Lowe (1943), is one in which the ingredients are well blended. It has a fine grain, thin cell walls, a smooth crumb, and a velvety feel when tasted. Undermixing, according to Nason (1939), results in a coarse, uneven texture and a cake which will stale rapidly, while overmixing gives a smaller volume and a compact texture.

The present investigation was undertaken to study the effect of different methods of combining the ingredients of a simple cake (containing fat) on various characteristics of the batters and the baked cakes. The consistency and specific gravity of cake batters prepared in various ways was determined, and related to the shortness, compressibility, and eating quality of the finished cakes.

Materials and Methods

Cake Formula. The cake formula used throughout the study was as follows:

Ingredients	Quantity (g)	Approximate measures
Fat	75	⅜ cup
Sugar	200	1 cup
Cake flour	168	1¾ cups
Baking powder	10	2½ teaspoons
Salt	1.5	¼ teaspoon
Vanilla	—	1 teaspoon
Dried whole eggs	27	4 tablespoons, packed
Water	100	6⅔ tablespoons
Milk	162.7	⅔ cup

Ingredients were as nearly identical as possible. Dried eggs from the same lot, a high-ratio vegetable shortening available to institutional and commercial bakeries, finely granulated sucrose, high-grade cake flour, grade A pasteurized whole milk obtained fresh daily, a

combination sulfate-phosphate (sodium aluminum sulfate-phosphate) baking powder, distilled water, salt, and vanilla were used in all cakes.

When the dried eggs were reconstituted they were added to the water and mixed either by shaking in a bottle or by beating in a bowl. When the water was at room temperature, the eggs stood for 30 minutes before they were combined with the other ingredients. When water at 67°C was used, the eggs were readily dispersed and no hydration period was allowed.

Methods of Mixing. A total of 200 cakes was made using six general methods of mixing. Minor alterations were made in these methods to bring the total number to 20.

Cakes were made by each method five times on two different types of three-speed mixers. For purposes of comparison and discussion, the work was divided into two series. In Series I the mixing was done on a Kitchen-Aid mixer with steel bowls of five-quart capacity. This mixer had a wire whip which was used for whipping the eggs and a flat paddle used for all the other mixing. In Series II a "Model C" Hamilton-Beach mixer with a one-quart and a three-quart vitreous bowl was used; the smaller bowl was used for whipping the eggs because it was a more suitable size for the amount. The three-quart bowl was flatter and more shallow than the bowl used with the Kitchen-Aid mixer. However, with each mixer the bowls were of sufficient size to permit thorough mixing of ingredients. The amount of batter was kept constant throughout the study.

The methods of mixing were briefly as follows:

Cake-Mixer Method: Reconstituted eggs whipped on high speed, fat, sugar, and salt added. Creamed on medium speed to desired specific gravity. Sifted flour and baking powder added alternately with liquid, using low speed.

METHOD I. Eggs reconstituted in all of the water at 68°C.

METHOD II. Eggs reconstituted in ¾ of the water at 68°C, ¼ of the water added with milk.

METHOD III. Eggs reconstituted in all of the water at room temperature. Hot water bath at 65°–70°C used while whipping.

METHOD IV. Same as III except ¾ of the water used for reconstituting eggs, and the remaining ¼ added with milk.

Modified Cake-Mixer Method: Creamed fat, sugar, and salt (and water if so specified in any modification) on medium speed. Changed to low speed and finished mixing.

METHOD V. Eggs sifted with flour and baking powder, then added alternately with combined milk and water.

METHOD VI. Same as V except ¾ of the water added with milk, and ¼ added with creamed mixture.

METHOD VII. Same as V except ½ of the water added with milk, and ½ added with creamed mixture.

METHOD VIII. Same as V except ¼ of the water added with milk, and ¾ added with creamed mixture.

METHOD IX. Same as V except all of the water added with creamed mixture.

METHOD X. Eggs made into paste with ½ of the water and added to creamed fat, sugar, and salt mixture, ½ of the water added with milk.

METHOD XI. Eggs sifted with sugar and salt and creamed with fat, all water added with creamed mixture.

METHOD XII. Same as XI except ¼ of the water added with milk and ¾ added with creamed mixture.

METHOD XIII. Eggs reconstituted and added with milk alternately with flour mixture; ¾ of the water added with eggs and ¼ added with creamed mixture.

METHOD XIV. Eggs sifted with sugar, salt, and approximately ¼ of the flour, then creamed with fat. All water added with creamed mixture.

Four-Minute Method:

METHOD XV. Eggs reconstituted with ¾ of the water, ¼ of the water added with milk. Dry ingredients sifted together. Fat and ⅔ of the liquid added. Mixed 1 minute on low speed, then 1 minute on medium speed. Remaining liquid and reconstituted eggs added. Mixed 1 minute on low speed and 1 minute on medium.

Modified "Conventional Sponge" Method:

METHOD XVI. Eggs reconstituted in all of the water at 68°C. Fat and ½ of the sugar creamed, low speed. Reconstituted eggs and other ½ of the sugar whipped 1 minute on low speed and 4 minutes on high speed. Changed to low speed to complete mixing. Baking powder, salt, and 1 tablespoon flour sifted together, added to fat-sugar mixture. Mixed slightly. One tablespoon of liquid added. Mixed. Remaining flour and liquid added alternately. Egg-sugar mixture added.

Conventional Method:

METHOD XVII. Eggs reconstituted in all of the water. Fat creamed on high speed. Sugar added, creamed on medium speed. Reconstituted eggs added and creaming continued on medium speed to desired specific gravity. Changed to low speed; flour, salt, and baking powder sifted together and added alternately with liquid.

Modified Conventional Method:

METHOD XVIII. Same as conventional except that eggs were sifted with sugar and salt and then creamed with fat. All of the water added with creamed mixture.

Flour-Batter Method:

METHOD XIX. Eggs reconstituted in all of the water at 68°C. Fat, flour, salt, and baking powder blended on low speed. Reconstituted eggs and sugar whipped on high speed to give a meringue-like mixture. Meringue and liquid added alternately to fat-flour mixture, ½ at a time, using low speed.

Dough-Batter Method:

METHOD XX. Eggs sifted with flour, salt, and baking powder, then blended with fat. All of the water added with milk. Sugar mixed with ½ of liquid and added ⅓ at a time. Remaining liquid added ½ at a time. In Series I, with Kitchen-Aid mixer, low speed was used throughout. In Series II, with Hamilton-Beach mixer, medium speed was used while incorporating sugar and ½ of the liquid; low speed used the remainder of the time.

For all cakes in the two series, the speed of mixing was the same except as specified in Method XX.

When the first mixture was of fat and sugar with or without added egg and water, it was usually creamed to a definite specific gravity. Alexander (1931) found that this reduced creaming and mixing variables.

Tests on Batters. The specific gravity determinations were made on the batters by filling a 57 ml aluminum cup, weighing it, and computing the ratio between the weight of the batter and an equal volume of water. The consistency of the batter was determined by putting the above cup of batter into a glass funnel, on the stem of which were two marks 5.0 cm apart. The bore of the stem was approximately 8 mm in diameter. The time necessary for the batter to move from

the upper mark to the lower one was recorded as the measure of consistency.

Baking. The batter remaining after the small cupful was removed was poured into an aluminum cake pan 7¾ inches square and 1⅞ inches deep and baked at 365°F (185°C) for 43 minutes in a gas oven equipped with a revolving hearth and an accurate thermostatic heat control.

Evaluation of Cakes. Approximately 20 hours after baking, the cakes were cut. Four uniform slices were cut using an apparatus similar to a miter box. The first slice was not used for testing because of the crust on one side.

The height of each cake was determined by measuring in centimeters the height of the fourth slice cut, at the outer edges, at the center, and at points one-half the distance from the center to each edge. The average of these five measurements was then recorded as the standing height of the cake.

A gram shortometer, an apparatus consisting of a modified spring balance and a remodeled laboratory balance, was used to measure in grams the force necessary to break a slice of cake one inch in thickness. This device was a modification of the one used by Bailey (1934) and was described by Kramer (1935).

After testing for shortness, one-half of each broken slice was used for testing the compressibility of the cake. The apparatus used was similar to that described by Platt and Kratz (1933).

The quality of the cake was determined by a palatability committee using a score card similar to that given by the Institute of American Meat Packers (1934).

Results

Comparison of Results with Two Mixers. The time of creaming was affected by the temperature. The specific gravity of the creamed mixture differed with the ingredients used. Variation in total time of mixing (Tables I and II) for the two mixers was largely due to difference in efficiency of the mixer in the first creaming. The Kitchen-Aid mixer with a paddle similar to that used on larger commercial models apparently incorporated a given amount of air in certain combinations of ingredients more quickly than did the household-type of mixer. In other instances the reverse was true.

Comparison of the average specific gravity of batters made by the same method with the two mixers shows that in roughly one-half of the cases the Kitchen-Aid mixer (Series I) gave the lighter batter and in the other half the Hamilton-Beach (Series II). This variation is difficult to explain.

The consistency of the batters also varied in the two series (Tables I and II), but methods which gave a thick batter with one mixer also gave a thick batter with the other.

On the whole the cakes mixed with the Kitchen-Aid mixer resembled "bakery-type" cakes. Although the volume was almost equal to that obtained in Series II the texture was more compact. The comments made by the judges indicated that they considered many of the cakes to be too firm and lacking in velvetiness. Some felt harsh and bready, others were tender but tended to crumble excessively. Shortness and compressibility tests (Tables I and II) show these cakes tended to be less tender and less compressible.

The cakes in Series II were more like the typical "homemade" product. They were less compact, had a fine even grain, and were tender, but held together with little tendency toward excessive crumbling. On the whole they were smooth, soft, and velvety to the touch and on the tongue. The cakes in this series were more compressible and more tender than those in Series I, due, no doubt, to the fact that the "homemade type" was less firm and compact.

The palatability committee indicated a preference for the "homemade type" of cake as is shown by the slightly higher scores given those cakes. The cakes in Series I, the "bakery type," were given a wider range of scores than those in Series II.

Although the cakes varied considerably more in quality than is indicated by the range in palatability scores, all were edible. The palatability scores included a number of factors such as crust, shape, color of crumb, moistness of crumb, tenderness, velvetiness, and eating quality; so in many instances a lower score on one point was offset by a higher score on another. By and large, the score for eating quality tended to parallel the total palatability score. Comparison of the two series shows that the means for eating quality were exactly the same. However, if all results are considered, it is evident that no method of mixing produced cakes of identical quality when the two mixers were used. This difference may have been due to variation in beater, shape of bowl, and/or number of revolutions per minute of the beater.

Comparison of Methods of Combining Ingredients. The primary purpose of this study was to compare methods of combining the ingredients in a simple cake containing fat. These results are summarized in Tables I and II.

Each method tended to give a characteristic batter, ranging from one so thin that it could be easily poured to one so viscous that it needed to be pushed into the corners of the baking pan. The difference in consistency was determined by the time required for a given

volume of batter to flow through a distance of 5.0 cm in the stem of a funnel. This varied from 0.2 minute for the thinnest batter to 52.8 minutes for the thickest. Tables I and II give averages for the different methods.

TABLE I

SERIES I. EFFECT OF METHOD OF MIXING UPON CHARACTERISTICS OF BATTER AND BAKED CAKE

Method		Batter				Baked cake			
		Time of mixing	Specific gravity	Final appearance	Consistency	Standing height	Shortness	Compressibility	Palatability score[1]
		min			*min*	*cm*	*g*	*mm*	
Cake-mixer	I	16.0	.8421	Fairly thick	10.4	5.2	125	4.3	57.8
	II	13.0	.8035	Fairly thick	11.6	5.3	124	4.3	57.8
	III	15.0	.8316	Medium thick	5.1	5.3	129	3.9	56.8
	IV	13.0	.8228	Medium thick	4.3	5.1	125	4.0	57.9
Modified cake-mixer	V	23.0	.8000	Thick[2]	17.6	5.0	112	3.8	56.9
	VI	23.0	.7632	Thick[2]	24.3	5.0	118	3.5	57.6
	VII	22.0	.7509	Thick[2]	29.2	5.0	123	3.5	57.3
	VIII	24.0	.7526	Thick, dull[2]	25.5	5.1	114	3.8	58.0
	IX	25.0	.7526	Thick[2]	28.5	4.9	120	3.6	57.4
	X	16.0	.8263	Thin	2.1	5.3	139	3.9	58.2
	XI	16.0	.8474	Very thin, glossy	0.3	5.3	141	3.6	57.9
	XII	16.0	.8614	Thin, glossy	0.3	5.1	147	3.4	56.8
	XIII	21.0	.7597	Thick, viscous	21.7	5.2	108	4.2	59.0
	XIV	16.0	.8667	Thin	0.3	5.5	141	3.4	56.8
Four-minute	XV	4.0	.9667	Very thin	0.5	4.6	113	3.1	54.9
Modified "conventional sponge"	XVI	8.4	.8667	Thin, slightly curdled	3.4	4.9	109	3.5	55.8
Conventional	XVII	15.7	.7649	Thick, dull	12.3	5.3	104	4.9	58.9
Modified conventional	XVIII	12.7	.7825	Thin, dull	0.7	5.4	119	3.9	58.2
Flour-batter	XIX	20.0	.9105	Medium thick	6.0	5.0	127	3.3	58.0
Dough-batter	XX	12.0	.9579	Quite thin	0.4	5.1	134	3.6	58.0

[1] Possible score 70.
[2] Curdled with first additions of liquid but became quite smooth, thick, and viscous with last portions of flour.

As a rule, when the eggs were sifted with the flour the batter was very thick. When the eggs were reconstituted before being added, the batter was generally thick. Exceptions were the Cake-Mixer Methods, in which the eggs were set in a hot water bath while whipping, and the Four-Minute Method. When the eggs were added with the sugar, a thin batter was usually obtained. This difference in consistency agrees with observations of Lowe and Nelson (1939), who found that the method of combining the ingredients affected the viscosity of the batter.

It was thought that temperature might be an important factor in determining the consistency of batters mixed by the different methods. However, the correlation between temperature and consistency was not significant (Table III).

Several batters curdled when the first portion of liquid was added (Tables I and II) but the curdled appearance disappeared and the

TABLE II

Series II. Effect of Method of Mixing upon Characteristics of Batter and Baked Cake

Method		Batter				Baked cake			
		Time of mixing	Specific gravity	Final appearance	Consistency	Standing height	Shortness	Compressibility	Palatability score[1]
		min			*min*	*cm*	*g*	*mm*	
Cake-mixer	I	20.0	.7930	Fairly thick	13.8	5.2	115	4.5	58.0
	II	16.0	.8211	Fairly thick	7.9	5.3	115	4.4	58.9
	III	21.0	.8439	Medium thick	1.7	5.3	127	4.3	56.9
	IV	18.0	.8578	Medium thick	2.0	5.1	135	3.6	57.1
Modified cake-mixer	V	21.0	.8351	Thick[2]	6.7	5.0	102	5.0	60.1
	VI	19.0	.7772	Thick[2]	22.0	5.1	103	5.7	59.0
	VII	20.0	.7684	Thick[2]	14.2	5.1	104	5.8	58.8
	VIII	21.0	.7579	Thick, dull[2]	28.7	5.1	97	6.6	60.3
	IX	21.0	.7579	Thick[2]	26.5	5.0	104	5.0	59.5
	X	14.0	.7965	Thin	7.2	5.3	114	4.7	59.2
	XI	16.0	.8386	Very thin, glossy	0.5	5.3	135	4.1	57.2
	XII	15.0	.8526	Thin, glossy	0.5	5.3	134	3.8	57.1
	XIII	21.0	.7667	Thick, viscous	22.3	5.1	101	4.9	58.1
	XIV	17.0	.8351	Thin	0.7	5.2	131	4.0	57.8
Four-minute	XV	4.0	1.0298	Very thin	0.2	4.6	105	3.1	55.7
Modified "conventional sponge"	XVI	10.4	.9456	Thin, slightly curdled	1.3	4.7	115	3.1	56.5
Conventional	XVII	16.7	.7684	Thick, dull	13.1	5.2	108	4.2	58.1
Modified conventional	XVIII	11.7	.7947	Thin, dull	1.3	5.3	116	4.1	58.0
Flour-batter	XIX	18.0	.9053	Medium thick	3.9	5.0	131	3.5	57.8
Dough-batter	XX	12.0	.9158	Quite thin	0.4	5.1	132	3.5	58.5

[1] Possible score 70.
[2] Curdled with first additions of liquid but became quite smooth, thick, and viscous with last portions of flour.

TABLE III

Correlation Coefficients for Selected Data Obtained from Batters and Baked Cakes[1]

Correlation between	Coefficient of correlation (r)	
	Series I	Series II
Specific gravity and consistency	−.66**	−.60**
Specific gravity and palatability	+.13	−.49**
Shortness and compressibility	−.54**	−.61**
Compressibility and palatability	+.16	+.71**
Shortness and palatability	−.33**	−.42**
Consistency and palatability	−.03	+.39**
Consistency and shortness	−.30**	−.54**
Consistency and compressibility	+.01	+.52**
Index to volume and specific gravity	−.21*	−.50**
Compressibility and specific gravity	−.27**	−.67**
Consistency and temperature of batter	+.19	−.10
Eating quality and palatability	+.64**	+.81**
Eating quality and other palatability factors	+.74**	+.72**
Specific gravity and shortness	+.26**	+.33**
Index to volume and consistency	−.29**	+.00
Index to volume and palatability	+.38**	+.24*
Eating quality and compressibility	+.25*	+.54**

[1] 100 cakes in each series. Calculations according to Snedecor (1940).
* Significant values.
** Highly significant values.

batter became smooth and thick when the dry ingredients and remaining liquid were added. The Modified "Conventional Sponge" Method resulted in a curdled finished batter. Some batters are described as "thick and dull" and others as "very thin and glossy." This difference in appearance may possibly be explained by the work of Collins and Sunderlin (1940), who found that the viscosity of the batter varied with the type of emulsion when the proportion of ingredients was constant. Thin batters, these workers believed, were associated with oil-in-water emulsions and thick ones with water-in-oil emulsions. Lowe (1943) states that in addition to these two types of emulsions, fat may be distributed in the cake as films, pools or lakes, or absorbed on the starch and proteins of the crumb; it may be at the cake/air interface; or it may be distributed by a combination of all or some of these. A microscopic examination of the batters in this study showed differences in the way in which the ingredients were distributed and in the number, size, and distribution of the air cells. However, it was not possible to classify these batters on the basis of type of emulsion or method of distribution of fat.

Relationship between Physical Properties of Batter and Cake Characteristics. An endeavor was made to find a correlation between certain physical properties of the batters and characteristics of the baked cakes (Table III). A highly significant negative correlation was found between specific gravity and consistency. This might indicate that the amount of air incorporated in the batter may be an important factor in determining the consistency of the batter. In the literature, emphasis has been placed upon the distribution of the fat in the batter as the factor important in determining consistency. The amount and distribution of the air seems to have been ignored.

The only other factor tested which correlated with consistency in both Series I and II was shortness. Here a highly significant negative correlation was found.

Undoubtedly the variation in specific gravity in the batter was dependent upon the amount of air incorporated in it. This ranged from 0.7368 to 1.0351 for the individual cakes and from 0.7509 to 1.0298 for different methods. Specific gravity apparently was closely related to physical characteristics of the cake. There was a significant negative correlation between specific gravity and index to volume in Series I and a highly significant negative correlation in Series II. There was a highly significant negative correlation between specific gravity and compressibility and a highly significant positive correlation between specific gravity and shortness. Thus it would appear that a light well-aerated batter is much more likely to produce a cake with desirable physical characteristics than is a heavy one. It would

also seem that even though specific gravity and consistency are highly correlated, specific gravity is a better indication of cake quality than is consistency.

The standing height of the baked cakes which was used as an "index of volume" varied from 4.6 to 5.5 cm. Roughly this represented a range from 1750 cc to 2090 cc in volume, a considerable difference. The smallest cakes were made from thin batters, but interestingly enough the largest ones were also obtained from thin batters. This is not wholly in agreement with Berrigan (1937) who found that cake batters having a low viscosity produced cakes of small volume and poor texture. As previously mentioned, there was a correlation between specific gravity of the batter and volume, but in Series I it was just significant. In Series I there was a highly significant correlation between "index of volume" and palatability and in Series II a significant one. Thus it would appear, as the commercial baker frequently asserts, volume is an important consideration to many consumers of bakery goods.

Shortness, the ease with which a slice of cake can be broken, showed a highly significant correlation with specific gravity. It showed a highly significant negative correlation with consistency, with compressibility, and with palatability. Compressibility was apparently not as dependable a measure of quality as was shortness. Only in Series II was there a highly significant correlation between compressibility and palatability. When only eating quality was considered there was a correlation with compressibility in both series, although it was highly significant only in Series II. Lowe (1943) has reported that the degree of compressibility varies with the method of mixing. Cakes mixed for a short period had a coarse, loose, porous texture, and were more compressible than cakes mixed for a longer time.

As already mentioned, the palatability committee evaluated a number of characteristics as well as eating quality. The correlation between eating quality and other factors scored by the committee and between eating quality and total palatability score was very high (Table III). Frequently, however, the comment was made that a cake which was otherwise of high quality "balled up" in the mouth. Hence, a cake which scored high in other characteristics might be marked down on eating quality because of the feel in the mouth.

Summary

Batters with a wide range in consistencies can be made from the same ingredients by varying the method of combining the ingredients.

Consistency and specific gravity were found to be closely related,

but consistency seemed to be a less reliable indication of the quality of the finished cake than did specific gravity.

Cake batters of low specific gravity tended to give cakes of good volume which were tender and compressible.

Very quick methods of mixing gave inferior cakes, but those methods requiring the most time did not necessarily give the best results.

Shortness and compressibility showed a highly significant correlation, but shortness appeared to be a better index to quality of the cake than was compressibility.

Two physical characteristics, index-to-volume and shortness, were correlated with the palatability score.

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STUDY OF SOME FACTORS INFLUENCING THE OXIDATION OF THIAMINE TO THIOCHROME

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Various methods of carrying out the ferricyanide oxidation of thiamine to thiochrome are recorded in the literature. Jansen (1936), who originally suggested this reaction as a method of estimation of thiamine, described three procedures, one for pure thiamine, another for the international adsorbate, and a third for yeast extract. The order of addition and the concentration of his reagents varies. Hennessy and Cerecedo (1939) used another concentration of alkali and a different quantity of sample, and directed that the ferricyanide be added before the alkali. Harris and Wang (1941) added the reagents in this order, but claimed only 50% recovery of thiamine unless methanol was added. The method given in *Cereal Laboratory Methods* (4th ed., 1941) published by the American Association of Cereal Chemists is essentially the method of Hennessy and Cerecedo (1939), in which the addition of the ferricyanide is made before that of the alkali.

Nicholls, Booth, Kent-Jones, Amos, and Ward (1942), Wang and Harris (1942), and Glick (1944) recommend the addition of the reagents in the reverse order, that is, the alkali before the ferricyanide, Glick recommending rapid addition from a pipette with large orifice. Pyke (1939) adds the sample to a mixture of methanol, alkali, and ferricyanide, but Booth (1940) claims incomplete oxidation of the thiamine by this premixing of reagents.

Conner and Straub (1941) and Merck and Company (1941) recommended a simultaneous addition of alkali and ferricyanide by the use of a premixed reagent. This procedure was also advised by the Research Corporation Committee (1941) and by Hoffer, Alcock, and Geddes (1943). In the two latter papers mention is made that the reagent should be added rapidly.

Andrews (1944) compared results of collaborative tests made by several of these procedures, after a canvass of 42 laboratories to determine the weaknesses in the method. It was found that the concentration of ferricyanide and the order of adding ferricyanide and alkali had some effect upon the assay, and that it was preferable, particularly where a considerable excess of ferricyanide was employed, to add the alkali first followed immediately by the ferricyanide.

The quantities of ferricyanide recommended in these methods vary rather widely. Conner and Straub (1941) found that such variations produced very little effect on fluorescence if optimal amounts of sodium hydroxide were present, while other workers, e.g., Hoffer *et al.* (1943) record considerable quenching of the fluorescence with increased amounts of ferricyanide.

The clarification of isobutanol for reading in the fluorometer is carried out in a number of ways, but most commonly by the use of anhydrous sodium sulfate. Some workers specify the quantity to be used, but generally the amount recommended is somewhat uncertain. Brown, Hamm, and Harrison (1943) reported that certain samples of sulfate imparted fluorescence to the isobutanol and warned against inaccuracies from this source.

Early experience in our laboratory with the thiochrome method had proved disappointing, as consistent results could not be obtained. Efforts to improve the replicability of the test led to a suspicion that the main difficulty was connected with the oxidation step and some of the various procedures for carrying out this step were therefore submitted to a critical study.

Experimental

All strengths of sodium hydroxide solution used are referred to as percentages by weight unless otherwise stated. Details of the oxidation procedures used will be described later. After oxidation, 15 ml of isobutanol were added, the mixture was shaken for 1 minute, and then centrifuged. The aqueous layer was rejected and the isobutanol cleared with 1 ml of ethanol. Its fluorescence was then read from the potentiometer scale of a Model 11 Coleman Spectrophotometer with mercury vapor lamp and other attachments.

Greater fluorescence was invariably obtained when the sodium hydroxide was added before the potassium ferricyanide than was the case when the order was reversed, yet sodium hydroxide itself is known to destroy thiamine. When 5 ml of neutral thiamine solution and 3 ml of 15% sodium hydroxide solution are used, the resulting mixture is about 1.5 *N* with respect to sodium hydroxide. Tests were made in which smaller quantities of 15% sodium hydroxide solution were added to 5 ml aliquots of standard solution containing 1 μg thiamine and 1 mg of ferricyanide. Normal fluorescence readings were obtained with 2 ml and 1 ml of sodium hydroxide solution, but with 2 drops of sodium hydroxide solution the readings were very low, and were not increased by the addition of 3 ml of sodium hydroxide solution a few seconds after the 2 drops had been added. No increase of fluorescence occurred either, if after adding the 2 drops of sodium hydroxide

solution, the solution was then made slightly acid before adding 3 ml of the alkali.

Buffer Experiments. To study this reduction of fluorescence more closely, oxidations were made at various pH levels using Sörensen's glycine buffers. Standard thiamine solution, 1 μg in 0.5 ml, was added to 4.5 ml buffer containing 0.9 mg of ferricyanide, followed 2 minutes later by 3 ml of 15% sodium hydroxide solution. Similar exposure was also made to the buffer alone for 2 minutes, the alkali then being added, followed immediately by the ferricyanide. The results are shown in Figure 1.

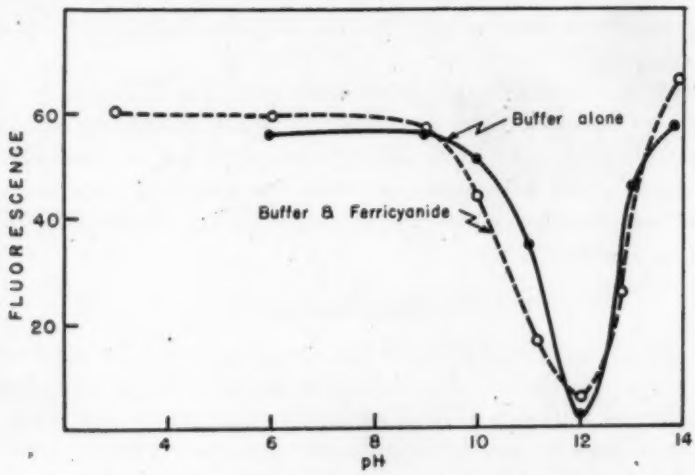

Fig. 1. Effect on fluorescence of exposure of thiamine for 2 minutes to buffer solutions at different pH levels, before and after addition of ferricyanide. Fluorescence values were obtained from potentiometer drum, blank deducted, using Coleman Model 11 Spectrophotometer set at 80 with quinine sulfate (0.27 μg/ml).

The general shape of the curves was confirmed in the absence of buffer by adding increasing amounts of very dilute alkali to the thiamine solution and checking the pH.

Since only a small amount of alkali is required to carry an unbuffered solution through the pH range shown in Figure 1, the thiamine solution was next exposed for 2 minutes to varying amounts of 15% sodium hydroxide solution together with ferricyanide. At the end of the 2 minutes, sufficient alkali was added to bring the total amount up to 3 ml and the fluorescence measured (Figure 2).

The dotted part of the curve represents data obtained with buffers to give a clearer idea of the results obtained at the different levels of alkalinity using 0 to 3 ml sodium hydroxide solution. During an actual oxidation, a variable loss of fluorescence from the point marked A seems probable, depending on speed of addition of the alkali and whether the mixture is agitated or not.

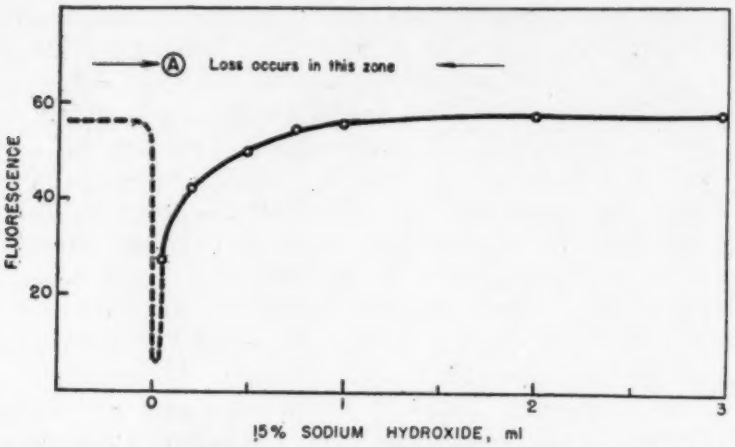

Fig. 2. Effect on fluorescence of exposure of thiamine for 2 minutes to increments of 15% sodium hydroxide solution, in presence of ferricyanide. Fluorescence values from drum of Coleman Model 11 Spectrophotometer, blank deducted, set at 80 using quinine sulfate (0.27 μg/ml).

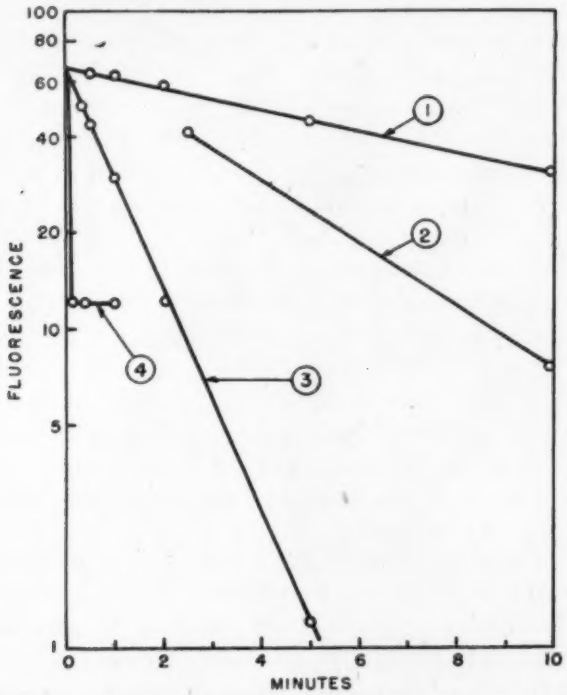

Fig. 3. Effect on fluorescence of exposure of thiamine for varying lengths of time to different strengths of alkali. Comparison with effect using buffer at pH 12 with ferricyanide. Fluorescence values from drum, Coleman Model 11 instrument, blank deducted, set at 80 with quinine (0.27 μg/ml), are plotted on a log. scale.

Curve 1.	3 ml 15% NaOH by wt.	Room temp. 25°C.[1]
Curve 2.	3 ml 30% NaOH by wt.	Room temp. 21.5°C.[1]
Curve 3.	1 drop 0.1 *N* NaOH	Room temp. 23°C.[1]
Curve 4.	Alkaline buffer (pH 12) and ferricyanide.	Room temp. 23°C.

[1] Room temperature differed somewhat for each curve, but the order of the curves and the sharp drop of curve 3 was confirmed by repeating certain points on each curve at a common temperature.

Effect on Fluorescence of Exposure of Thiamine to Alkali of Different Strengths, for Varying Lengths of Time. The similarity of the curves in Figure 1 shows that mild alkali alone has much the same effect on fluorescence as when mild alkali and ferricyanide are used. To study these effects, three series of experiments were carried out at room temperature. In two of these, 5 ml of thiamine solution (pH about 6) were exposed to 3 ml of 30% and 3 ml of 15% sodium hydroxide solution respectively for various time intervals up to 10 minutes, and ferricyanide was then added. In the third series, very weak alkali was used, 1 drop of 0.1 *N* sodium hydroxide solution acted on 5 ml of thiamine solution for the time stated, after which 3 ml of 15% sodium hydroxide solution was added, followed rapidly by the ferricyanide. The results are shown in Curves 1, 2, and 3 of Figure 3.

Action of the very weak alkali (Curve 3) is more rapid than that of the 15% or the 30%, the fluorescence falling nearly to zero in 5 minutes. Of the stronger solutions, the 30% alkali (Curve 2) acted more rapidly than the 15% alkali (Curve 1). The order of these curves indicates that in weak alkali, action may proceed along a different path from that in the strong alkali. Tests were made to see if thiamine was actually destroyed when acted on by weak alkali. After allowing thiamine solution to be acted upon by 1 drop of 0.1 *N* alkali for 5 minutes (corresponding to the lowest point on Curve 3), 2 drops of 0.1 *N* acid were then allowed to act for the same length of time, before adding 3 ml of 15% alkali and ferricyanide. Almost all of the lost fluorescence was restored. In contrast, in the earlier experiment in which ferricyanide was present during the mild alkaline treatment, no reversibility was obtained after acidifying.

Curve 4, Figure 3, is inserted to show how much more rapid is the effect produced when ferricyanide and mild alkali both act together. Thiamine added to a mixture of ferricyanide and buffer of pH 12 for only 10 seconds before addition of 3 ml of 15% alkali resulted in a striking loss of fluorescence which remained the same when repeated at 20- and 60-second intervals.

Comparison of Fluorescence Obtained by Commonly Used Methods. The three methods of oxidation commonly in use, one in which the ferricyanide is added before the alkali (Method 1), a second in which the order is reversed (Method 2), and a third in which these reagents are premixed before making the addition (Method 3), were compared using 5 ml of thiamine solution containing approximately 1 μg, 3 ml of 15% sodium hydroxide solution, and an oxidation interval of 30 seconds. The manner of making the additions was varied by pouring both slowly and rapidly with and without agitation.

A fourth method (Method 4a) based on the experiments reported in this paper was also examined. In this method the thiamine solution was added to the reagents, rather than adding the reagents to the sample. The freshly made, mixed reagent was kept well agitated during the addition from a pipette (draining in about 15 seconds) of the 5 ml of thiamine solution. From this point, the procedure was the same as for the others.

A slight modification of this procedure (Method 4b) was introduced by pouring the thiamine solution into the alkali, instead of into a mixture of alkali and ferricyanide, the oxidation then being carried out by the addition of the ferricyanide immediately after. These results are summarized in Table I.

TABLE I

COMPARISON OF FLUORESCENCE OBTAINED UPON OXIDATION OF THIAMINE BY VARIOUS METHODS

(Level of alkalinity is 3 ml of 15% sodium hydroxide)

Manner of addition	Reagent into thiamine			Thiamine into reagent	
	Method [1]			Method 4 [1]	
	1	2	3	a	b
	Fluorescence [2]				
Slow, with shaking	35.0	60.0	56.0	64.0	61.5
Slow, without shaking	47.5	61.0	57.5	64.0	61.5
Fast, with shaking	54.0	61.0	60.0	62.0	60.0
Fast, without shaking	57.5	61.5	62.5	63.5	60.5
Mean	48.5	60.9	59.0	63.4	60.9

[1] Method 1. Ferricyanide added to sample before alkali. Method 2. Alkali added first, followed by ferricyanide. Method 3. Mixed reagent added to sample. Method 4. (a) Sample poured into mixed reagent. (b) Sample poured into alkali, followed by ferricyanide.

[2] Fluorescence values obtained from potentiometer drum, blank deducted, using Coleman Model 11 Spectrophotometer set at 80 with quinine sulfate (0.27 μg/ml).

Adding the ferricyanide before the alkali (Method 1) gave low and variable results. By reversing the order (Method 2), higher and more uniform results were obtained, whereas the mixed reagents (Method 3) gave intermediate values. Method 4a gave the highest fluorescence with small variability, while Method 4b showed results similar to those of Method 2.

Since the amount and strength of alkali, namely 3 ml of a 15% solution, are so commonly used, it might be supposed that this quantity produces maximum fluorescence. Tests indicated, however, that this was not the case. Using procedure 4a, 5 ml aliquots of thiamine solu-

tion were poured into 3 ml of alkali of higher strengths, but containing the same amount (1 mg) of ferricyanide. The following results were obtained.

Sodium hydroxide % by wt.	Fluorescence as % (approx.)
15	100
30	114
36	118
42	118
48	118

Three ml of 36% by weight sodium hydroxide solution gave a maximum value.

Effect of Varying the Amount of Ferricyanide. A brief study was made of the effect on fluorescence of using differing amounts of ferricyanide. Several series of tests using 1 μg of thiamine in 5 ml of solution were carried out using different methods of oxidation with the results shown in Table II.

TABLE II

EFFECT ON FLUORESCENCE OF VARYING AMOUNTS OF FERRICYANIDE

	Fluorescence as percentage of maximum		
Ferricyanide added	Method 3	Method 4a [1]	Method 4b
mg			
0.1	100	100	100
0.3	94	—	—
0.5	92	—	100
1.0	87.5	100	100
2.0	82	100	100
3.0	—	100	98
4.0	—	97.5	95
5.0	70.5	—	92
8.0	—	94.5	—
10.0	69	—	90.5

[1] 33% alkali used; other methods 15%.

The most desirable method to use in assays where extraneous oxidizable material may be present is one permitting considerable latitude in the amount of ferricyanide added without affecting fluorescence. On this basis Method 4a using 33% alkali, or Method 4b using 15% alkali, gives satisfactory results.

Fluorescence Attributable to Anhydrous Sodium Sulfate. Early in the course of these studies erratic results were occasionally obtained, and this difficulty was traced to the presence of fluorescent material in the sodium sulfate. A study of this aspect of the method was therefore made.

Four lots of one brand, together with two other brands, all well known and of analytical grade, were examined. Tests were made by

shaking with the isobutanol separated from alkali and water as in the usual blank test. Fluorescence was measured after centrifuging to remove any tiny crystals stated in Merck and Company's method (1941) to enhance fluorescence. Two strengths of quinine were used to obtain instrument settings corresponding to those employed when measuring 0.5 μg and 1.0 μg of thiamine. One lot of brand number 1 giving the highest reading was tested again after extraction with isobutanol and ether. The results obtained are shown in Table III, blank readings on the isobutanol being deducted in each case.

TABLE III

VARIATION OF FLUORESCENCE OBTAINED FROM DIFFERENT SUPPLIES OF ANHYDROUS SODIUM SULFATE

		Fluorescence [3]	
Brand No.	Lot No.	0.5 μg setting	1.0 μg setting
1	(a)	17.5	9.7
	(a) [1]	5.5	3.0
	(b)	6.5	3.5
	(c)	6.0	3.2
2	—	13.5	7.3
3	—	8.0	4.3
1	(d) [2]	4.0–8.6	2.4–5.2

[1] Extracted with isobutanol and ether.
[2] This lot number examined at 2 and 4 g levels. All others at 3 g.
[3] Fluorescence units from potentiometer drum of Coleman Number 11 Spectrophotometer, blank deducted, set at 80 with quinine of two strengths (0.13 μg/ml and 0.27 μg/ml).

Wide variations were obtained in the blank values with the different lots of sodium sulfate. Part of this variation may be due to differences in the final pH of the isobutanol if varying amounts of water and alkali are extracted by different brands of sulfate, since Wokes, Organ, Still, and Jacoby (1944) showed that, with thiochrome at any rate, fluorescence varies with the pH of the isobutanol. Most of the variation must be due to fluorescent material present as impurity, since the extracted material is much lower than the unextracted.

Discussion

The comparison of the methods commonly in use for carrying out the oxidation of thiamine to thiochrome shows that when ferricyanide is added first to the sample, and then followed by alkali, low and uncertain results are obtained. If the alkali is added very slowly, a much lower result is obtained than if it is added rapidly. Even if alkali is added first, some lowering of fluorescence is produced if it is added slowly. Apparently a side-reaction takes place, the extent of which is

influenced by the rapidity with which the reagents are added. Where alkali alone acts, this side-reaction is reversible, and it seems possible that it is related in some way to the reversible change noted by Williams and Ruehle (1935) when titrating thiamine electrometrically with sodium hydroxide. At a certain point where peculiar behavior was observed, it was believed that some molecular rearrangement took place. When ferricyanide is added first, the side-reaction is very rapid, and it is no longer reversible.

The studies on the action of alkali during the course of the oxidation, involving the use of buffers and allowing the alkali to act for varying periods of time, show that a zone of low alkalinity coincides with a zone of low fluorescence and that this is more marked when ferricyanide acts at the same time as the alkali.

Because dilute alkali alone has no permanent effect on thiamine, as indicated by the fact that treatment with acid restores fluorescence, it has been suggested that the action of the ferricyanide may proceed along two paths, one in mild alkali, producing nonfluorescent, irreversible compounds, the other in strong alkali favoring optimum production of thiochrome. This would explain adequately those cases where ferricyanide is present during the action. Since the rapid loss of fluorescence by action of weak alkali reported in Figure 3, Curve 3, was obtained with an oxidation procedure at high alkalinity, it would seem that whatever changes had been produced in the thiamine by the weak alkali must still have persisted after being made strongly alkaline. A slow reversion of the thiamine to its former condition may take place. In discussing these observations with Williams,[1] the author's attention was called to a paper by Zima and Williams (1940) showing formulas for the free base (2), a carbinol form (3), and the sodium salt (4). Assuming that the thiochrome is formed by oxidation of the free base rather than the carbinol, the data given suggest that weak alkali favors rearrangement to carbinol, while strong alkali represses it. Suppression of thiochrome formation by still stronger alkali might be due to predominance of the sodium salt. Whatever the explanation, it is necessary during oxidation to avoid conditions favoring low alkalinity. When alkali is added to the sample or to the sample containing ferricyanide, as is done at present by many workers, the first few drops produce such conditions. Rapid addition will, of course, reduce them to a minimum, but standardization between laboratories may present difficulties.

The obvious way to overcome the difficulty is to pour the sample slowly with agitation into the alkali or mixture of alkali and ferri-

[1] Williams, R. R., Bell Telephone Laboratories, Murray Hill, New Jersey, private communication.

cyanide. By this means, the alkalinity is maintained at a high level throughout the addition, and the side-reaction is either prevented or held to a minimum. The use of still stronger alkali than 15% was found to be even more efficient in preventing this reaction.

To secure maximum fluorescence, the concentration of the alkali at the beginning and during the addition of sample is more important than the actual quantity of alkali. A study of the data of Conner and Straub (1941), and of our own results where maximum fluorescence was obtained, seems to indicate that to secure maximum fluorescence, an initial concentration of about 33% (45% by volume) of alkali and a final one of not less than about 11 or 12% are necessary. These conditions are met for a 5-ml assay sample by using 2 or preferably 3 ml of 33% sodium hydroxide solution. In their sodium hydroxide study, Conner and Straub (1941) added 1 ml thiamine solution to 1 ml of 45% by volume sodium hydroxide containing the ferricyanide, and only after oxidation was this diluted to 5 ml. These conditions were satisfactory for production of maximum fluorescence and meet the minimal values given above. In an actual assay, however, their order of addition is reversed, the concentrations are changed, and the conditions are no longer such as would necessarily give maximum values.

Experiments in which the quantity of ferricyanide was varied showed that the proposed method of pouring the sample into the reagent gave satisfactory results, a variation in amount from 0.1 mg to about 3 mg resulting in almost no lowering from the maximum fluorescence. Even with 8 or 10 mg of ferricyanide, the fluorescence decreased only a few percent. The results obtained when 33% alkali was used were better than those with 15%, the total drop in fluorescence, even with 8 mg being only about 5%.

While the use of alkali of the higher strengths has given satisfactory results on pure thiamine solutions and eluates, work on extracts obtained by shortened procedures such as that of Hoffer *et al.* (1943) may not give such favorable results. Experiments with various assay materials are necessary to ascertain the general utility of such a modification in procedure.

The variable blanks obtained with different lots of anhydrous sodium sulfate confirm the findings of Brown *et al.* (1943) that this material is not necessarily free from fluorescence. The author has discontinued its use, and prefers to add 1 ml of ethanol to the isobutanol for clearing purposes. Recovery of purified isobutanol for further use is easily made by distillation after giving two or three acid washings to remove ethanol.

Summary

When thiamine is oxidized to thiochrome by the commonly used methods, a loss of fluorescence occurs. The loss is minimized by adding the eluate slowly with agitation to a mixture of ferricyanide and 33% (45% by volume) sodium hydroxide solution. With this procedure the quantity of ferricyanide can be varied over a wide range without affecting the results.

Certain lots of anhydrous sodium sulfate, commonly used for clearing isobutanol extracts, contain fluorescent material and the use of ethanol is recommended in its place.

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THE PHOSPHORYLASE OF WAXY MAIZE [1]

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The enzyme phosphorylase which catalyzes the reversible reaction,

$$\text{starch} + \text{inorganic phosphate} \rightleftarrows \text{glucose-1-phosphate},$$

is a part of a complex system of enzymes which has to do with carbohydrate metabolism through phosphorylation. The mechanism formulated by Meyerhof (1935, 1941) is in part as follows:

glycogen, starch	d-glucose
$+H_3PO_4$ ⇵ (phosphorylase)	$+H_3PO_4$ ⇵ (hexokinase)
glucose-1-phosphate ⟷	glucose-6-phosphate
(Cori ester)	(Robison ester)
	⇵ (isomerase)
	fructose-6-phosphate
	(Neuberg ester)
	$+H_3PO_4$ ⇵
	fructose-1, 6-diphosphate
	(Harden-Young ester)

A study of this mechanism reveals the difficulties involved in attempting to isolate for study the one reaction of starch to glucose-1-phosphate.

The relationship among the principal reactions that may take place when glucose-1-phosphate is present in the enzyme system or when it is

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formed through the breakdown of starch by phosphorylase (Hanes, 1940a) is shown as follows:

(1) Glucose-1-phosphate is transformed progressively, by the enzymes indicated, to reducing hexose-6-phosphates (shown by Sutherland, Colowick, and Cori, 1941, to be reversible) with a resultant decrease in inorganic phosphate.

(2) Glucose-1-phosphate is transformed by phosphorylase into a polysaccharide and inorganic phosphate is liberated; the reaction may be catalyzed by the presence of a trace of starch.

(3) The polysaccharide formed (as in 2) is hydrolyzed by amylase (if present in the system) and thus the equilibrium may be shifted to the left.

(4) Hexose-6-phosphate (as in 1) is transformed into hexose-diphosphate by the appropriate enzymes with a consequent decrease in inorganic phosphate.

(5) When the concentration of glucose-1-phosphate falls to a certain level, reaction 2 ceases; further reduction of glucose-1-phosphate (by continuance of reaction 1) causes the reversal of reaction 2 and thus brings about the reconversion of the polysaccharide into glucose-phosphate.

It is possible to vary the conditions of the reactions and the preliminary treatments of the extract and thus to alter the relative velocities of these different reactions. Hanes (1940b) showed that at pH 8.0 to 8.5 reaction 1 proceeded very rapidly at the expense of reaction 2, which became negligibly small. The opposite effect—speeding up of reaction 2 and slowing down of reaction 1—was noted at pH 6.4.

Hanes (1940a), using potato extracts, studied the effect of pH and of the concentrations of the three reactants (starch, glucose-1-phosphate, and inorganic phosphate) upon the reversibility of the reactions:

$$\text{starch} + \text{inorganic phosphate} \rightleftarrows \text{glucose-1-phosphate}$$

He concluded (1) that the final equilibrium state is dependent upon the pH of the digest; and (2) that the final equilibrium state is independent of the concentrations of the reactants or the enzymes.

Hanes (1940) made purified phosphorylase preparations from pea seeds and showed that the enzyme acts on a variety of substrates; for example, saccharides composed of glucopyranose units linked in positions 1 and 4 (as in maltose), regardless of chain length and whether in colloidal aggregation or in free form terminated by reducing groups. Hanes concluded that the action of phosphorylase on starch and dextrins consisted of an endwise attack at the nonaldehydic end of the chain structure, with the liberation of individual glucose units in the form of glucose-1-phosphate. Hanes also reported a method for the

preparation of glucose-1-phosphate in good yield from starch, through the action of a partially purified phosphorylase from potatoes.

Cori, Cori, and Schmidt (1939) studied the polysaccharides synthesized through phosphorylase by the reaction:

$$\text{glucose-1-phosphate} \rightleftarrows \text{starch} + \text{inorganic phosphate}$$

They reported that the polysaccharide formed by phosphorylase from brain, heart, liver, and yeast gave a brown color with iodine and resembled glycogen, whereas the enzyme from muscle extract synthesized a polysaccharide that gave a blue color with iodine and resembled starch. Hanes (1940a) also reported a blue-staining polysaccharide synthesized by potato phosphorylase.

The studies on phosphorylase by Hanes (1940a), Cori, Colowick, and Cori (1938, 1938a, 1939), Cori, Schmidt, and Cori (1939), Cori and Cori (1943), and Green and Stumpf (1942) showed the presence of phosphorylase in a wide variety of animal and vegetable tissues, and indicated that a further study of the problem might reveal a correlation between the nature of the enzyme action in a plant tissue and the type of starch synthesized (amylose or amylopectin). Experiments indicate that waxy starches are amylopectin (Bates, 1943), and thus it is of interest to study the enzyme system of waxy maize. The experiments were planned (1) to obtain some estimate of the role of phosphorylase in the development of the waxy maize kernel, and (2) to prepare a phosphorylase enzyme concentrate from waxy maize, and (3) to study the polysaccharide formed by the action of waxy maize phosphorylase with glucose-1-phosphate.

Experimental Methods and Results

Phosphorus Determinations. By making use of the equilibrium equation, starch + inorganic phosphate $\rightleftarrows$ glucose-1-phosphate, the activity of the enzyme acting upon starch or glucose-1-phosphate was determined by measuring the decrease in inorganic phosphorus as shown by the forward direction of the reaction in which starch is the substrate, or the increase in phosphorus as indicated by the reverse reaction, in which glucose-1-phosphate is the substrate. Cori, Colowick, and Cori (1938) showed that the determination of inorganic phosphorus is justified as a procedure for measuring the formation or breakdown of glucose-1-phosphate, although the method reported by Hanes (1940) was followed in many experiments in which the free, or inorganic, phosphorus was compared with the hydrolyzed or 7-minute phosphorus determinations, representing total free + ester-phosphorus.

In all tests, the following procedure was used: An aliquot was removed from the enzymic digest and added to 5 ml of 10% trichloracetic

acid (to precipitate the protein). Centrifugation cleared the mixture and an aliquot of the centrifugate was used. To this sample were added 5 ml of acidic ammonium molybdate, 2 ml of 1-amino-2-naphthol-4-sulfonic acid, and water to 50 ml volume. A blue color developed which was read exactly 20 minutes after adding the reducing agent. The method was adapted from those described by Fiske and Subbarow (1925) and by King (1932). The reading was made by means of a photoelectric colorimeter, and translated to milligrams of phosphorus by means of a calibration curve. (Corning Glass filters, No. 246 Red, 4 mm were used. These transmit light in the region of 600 to 650 mμ.)

Activity of the Enzyme during the Growth of Waxy Maize. For these studies, the waxy maize was collected from the field and ground to a pulp (or flour). Distilled water equal to the weight of corn was added and the mixture held at icebox temperature (approximately 5°C) for 18 hours. (A small amount of thymol was added to retard the action of microorganisms.) The solid matter was discarded and the liquid enzyme extract cleared by centrifugation. To remove the remaining inactive material (protein and starch) the enzyme extract was heated rapidly to 50°C and kept at that temperature for 5 minutes and centrifuged. Ammonium sulfate precipitates were made by the following procedure: 20 g of ammonium sulfate per 100 ml of extract was added while the solution was still warm, and the precipitate was centrifuged off and discarded; from the centrifugate, another fractional precipitation with 16 g of ammonium sulfate per 100 ml of extract yielded active enzyme.

The enzyme precipitate was suspended in citrate buffer and used in digestion mixtures with glucose-1-phosphate (Cori, Colowick, and Cori, 1937) as a substrate. According to the method of Green and Stumpf (1942) the activity units were determined as the amount of enzyme which catalyzes the liberation of 0.1 mg of inorganic phosphate from glucose-1-phosphate in 3 minutes at 38°C and pH 6.0.

Tests for enzyme activity were made upon samples of waxy maize collected at short intervals (1 week to 10 days) during the season of development of the corn kernel. The data from these experiments are reported in Table I. Calculated on the basis of the units of phosphorylase per gram of dry corn, the activity varied from 5.09 to 0.28 units during the period from two weeks after pollination to maturity. The trend is the same if the data are reported on the basis of the units of phosphorylase per gram of waxy maize as collected from the field; on the latter basis the data show a variation of .85 to .26 units over the same period. Although enzyme concentrates of considerable activity

TABLE I

PHOSPHORYLASE ACTIVITY IN WAXY MAIZE AT DIFFERENT STAGES OF GROWTH

Days after pollination	Moisture content	Phosphorylase units per gram	
		as collected	dry weight
	%		
16	83.2	0.85	5.09
23	68.5	0.37	1.16
30	51.5	0.45	0.95
40	42.9	0.39	0.70
51	35.0	0.48	0.74
Mature corn	6.1	0.26	0.28

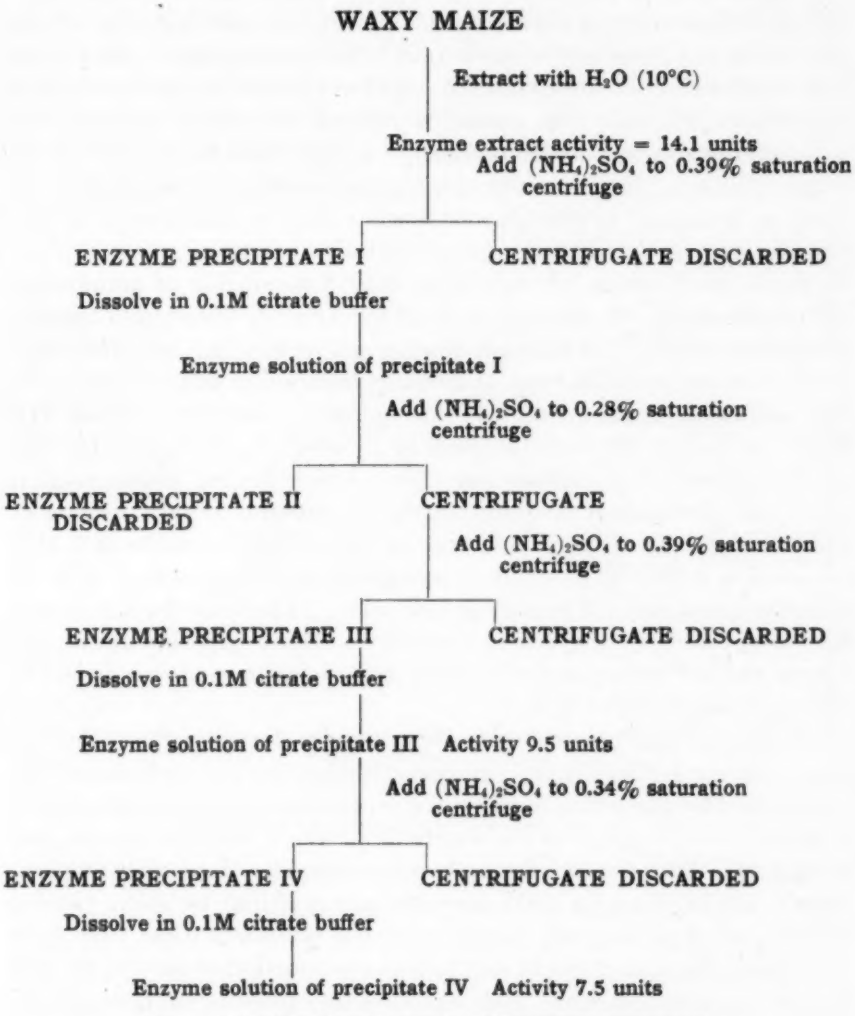

Fig. 1. A flow sheet showing steps in making enzyme concentrates.

could be obtained from corn during the later stages of growth, the data show a decrease in phosphorylase activity as the maize kernel develops.

Concentration of the Enzyme. A study was made of the methods for concentration of phosphorylase by dialysis, precipitation, and adsorption as used by Cori and co-workers (1938, 1938a, 1939), Hanes (1940), and Green and Stumpf (1942). An adaptation of these methods was used for the preparation of phosphorylase concentrates from waxy maize. Dialysis of the extract against distilled water was found to aid in forming protein precipitates which may be taken out, and to remove the inorganic phosphate originally present in the extract.

Figure 1 shows the procedure and the data obtained in making a phosphorylase enzyme concentrate. During the precipitation of the enzyme, it was necessary to work quickly and continuously, since there was deterioration of the enzyme if held for a period in the presence of ammonium sulfate. The results show that fractional precipitation with ammonium sulfate accomplishes a separation of inactive from active material, always retaining a high percentage of the original activity as measured in phosphorylase units (Green and Stumpf, 1942). Starting with an extract containing 14.1 units per 100 ml, the flow sheet indicates precipitation of enzyme at 0.39% saturation of ammonium sulfate, dissolving the precipitate in 25 ml of citrate buffer, and another fractional precipitation with ammonium sulfate in which inactive material is removed in precipitate II and the ammonium sulfate concentration increased again to 0.39% saturation to make precipitate III. When precipitate III is redissolved in 25 ml of citrate buffer, the dispersion shows 9.5 phosphorylase units, which finding indicates that 67.4% of the original enzyme activity is present in this dispersion. From this enzyme dispersion, a finer fractionation was made at 0.34% ammonium sulfate to give a final precipitate which, dissolved in 10 ml of buffer, contained 7.5 phosphorylase units. The final dispersion thus held about 53% of the original activity. Many experiments of this nature yielded corresponding results, and the dispersions were used in the experiments which follow.

A Study of the Polysaccharide Formed by the Action of Phosphorylase upon Glucose-1-Phosphate. An investigation of the polysaccharide synthesized by the action of waxy maize phosphorylase was undertaken to determine the nature of the synthetic product, and thus to compare it with the natural starch from the same source. Bates, French, and Rundle (1943) found that a potentiometric iodine titration yielded information regarding the structure of the polysaccharide. By such a titration, Bates *et al.* could estimate the proportion of each of the two starch fractions, amylose and amylopectin, present in the sample. When amylose or straight chain material was titrated, an iodine com-

plex was formed and the iodine activity remained about the same until the complex formation was completed (giving a straight line portion to the titration curve); then the potential began to rise sharply. In contrast, amylopectin showed a steady increase in potential throughout the titration.

This technique was applied to the analysis of the small amounts of synthetic polysaccharide formed in digests containing the waxy maize phosphorylase. The digests were made up to contain the potassium salt of glucose-1-phosphate, a catalytic amount of soluble starch, citrate buffer, and the enzyme concentrate of waxy maize. (A blank determination on the catalytic amount of starch was made.) Digestion was carried out at 38°C for one-half hour. At the end of this period, the digestion was stopped by the addition of 0.5 *N* potassium hydroxide, made up to 0.05 *N* with respect to potassium iodide, diluted to 100 ml, and titrated potentiometrically. The results of two experiments (Digests A and B) are shown in Figure 2, together with the

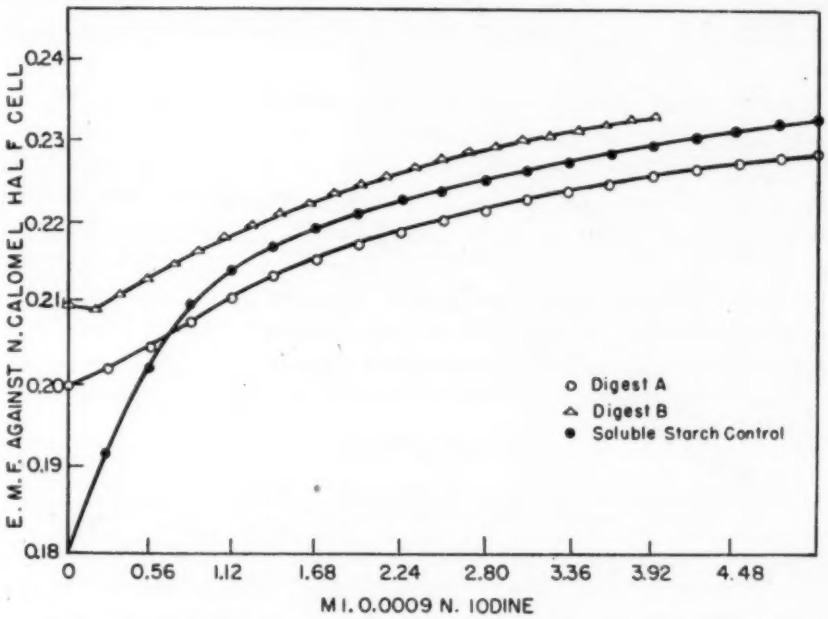

Fig. 2. Iodine titration curve of soluble starch control, and of enzyme Digests A and B.

curve for the soluble starch used as a catalyst. The soluble starch contains no amylose as the curve is like that of amylopectin. The digestion curves are of a different nature and resemble those of a straight chain material which binds iodine. The high potential of the curve indicates the formation of material of short-chain length.

In another experiment (Digest D), the enzyme dispersion was pre-

pared as previously described except that the original extract was dialyzed for 23 hours at 5°C (icebox temperature) against 0.2 *M* potassium chloride, as an additional means of purification. After dialysis, the phosphorylase was fractionally precipitated with ammonium sulfate.

This digest contained the phosphorylase which was precipitated at 0.28 to 0.39% saturation with ammonium sulfate, from 750 ml of extract; it was made up to a volume of 25 ml, to which was added 0.2 ml starch, 1 ml of 0.1 *M* potassium salt of glucose-1-phosphate, 3.8 ml of 0.1 *M* citrate buffer (making a volume of 30 ml). Digestion was carried out at room temperature. Aliquots for the determination of free-phosphorus, free-+ ester-phosphorus, and for iodine titration, were removed after 1.5 minutes, after 12 hours, and after 24 hours. The iodine titration curves are shown in Figure 3 together with that for soluble starch and a titration curve for 0.05 *N* potassium iodide solution.

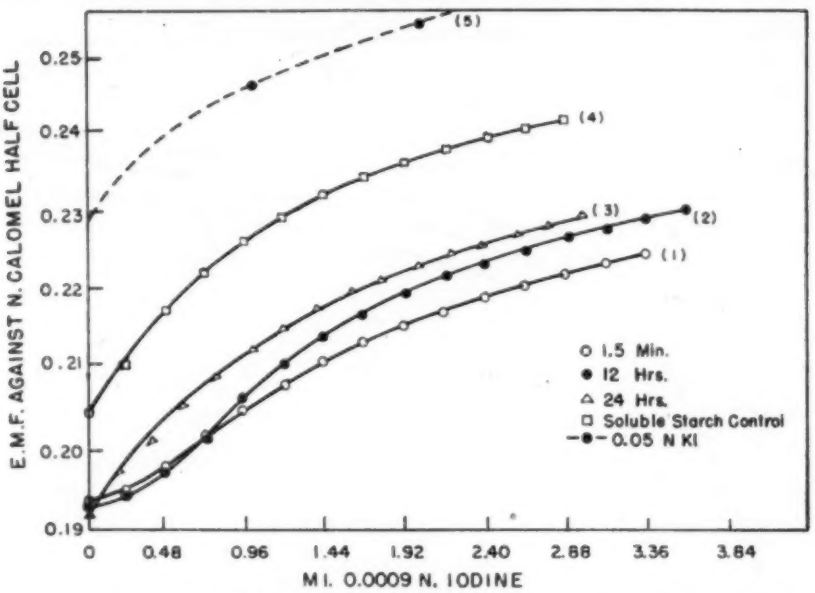

Fig. 3. Iodine titration curve of soluble starch control and of KI, together with curves for three time-interval aliquots of Digest D.

The phosphorus analyses showed an increase in inorganic phosphorus which corresponds to the synthesis of 14.3 mg of polysaccharide in the entire time of digestion. In the aliquots tested, at different time intervals, the following amounts of polysaccharide were indicated:

0	1.5 minutes	0.32 mg
0	12 hours	1.66 mg
0	24 hours	2.38 mg

The results of the iodine titration of the aliquots removed at different time intervals (Curves 1, 2, and 3, Figure 3) seem to indicate that up to 12 hours digestion at room temperature, straight chain iodine-binding material was formed, while a change in the character of the starch was apparent in the period from 12 to 24 hours, at which time the curve is of the nature of amylopectin.

These data are interpreted to indicate that the phosphorylase of waxy maize acting upon glucose-1-phosphate as a substrate liberates inorganic phosphorus with the synthesis of products of a starch-like nature, as indicated by the iodine-titration method.

Summary

Waxy maize collected from the field during growth from 16 days after pollination to maturity was tested by precipitation of the enzyme from extracts and determination of the phosphorylase units present. Data for the 1942 corn showed a considerable activity of phosphorylase in the earliest corn tested. Phosphorylase precipitates were obtained from corn at later stages of growth, and these showed decreasing activity up to maturity.

The techniques reported in the literature for the purification and concentration of the enzyme were tested to determine the method which would be most adaptable to the preparation of phosphorylase from waxy maize. Fractional precipitation with ammonium sulfate at concentrations between 0.28 and 0.34% of saturation yielded the most active precipitate.

Digests containing glucose-1-phosphate and phosphorylase of waxy maize were tested for phosphorus content, and by the iodine titration method. The data indicated the liberation of phosphorus with consequent formation of iodine-binding material of a starch-like nature.

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SOME EFFECTS OF CABINET FERMENTATION ON SPONGE TEMPERATURES AND DOUGH AND BREAD CHARACTERISTICS

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The use of cabinet fermentation has excited a great deal of interest among bakers and cereal chemists in recent years. Some have contended that great benefit is derived from the use of cabinets, within which the troughs containing the sponges are allowed to stand during sponge fermentation. Others have just as vigorously argued that the use of cabinets produces no significant changes in the sponge or in the resulting bread.

It has been our purpose to study cabinet fermentation in order to determine whether it is effective under bake shop conditions, and if so, to what extent. With this end in view, sponges were fermented in cabinets and in open troughs under varying conditions of temperature and humidity. Observations were made on sponge temperature, fermentation loss, pH of sponge, farinograph sponge and dough curves, handling of the doughs in the makeup machinery, proof time, loaf

volume, and general bread characteristics, such as texture, grain, and external appearance.

Formula and Procedure

A commercial type sponge-dough formula was used, including milk powder, malt extract, and bread improver. In the sponge 30 lbs. of flour were used, and in the dough 20 lbs.; the sponge flour was a Northwestern patent and the dough flour a Kansas patent; both flours were bleached.

The sponges were mixed at the temperatures indicated, placed in open or cabinet-enclosed troughs, and fermented $2\frac{3}{4}$ to $4\frac{1}{2}$ hours, usually in a controlled dough room at 80°F (26.7°C) and 96% humidity. The fermented sponge was then mixed with the balance of the dough ingredients. After a floor time of 15 minutes, the dough was put through the divider and rounder, through the molder 12 minutes later, then proofed at 96°F (35.6°C) and 90% humidity, and baked 30 minutes at 480°F (249°C) with steam in the oven.

Variations in formula or procedure were made as indicated.

Temperature measurements on some of the sponges were made with calibrated copper-constantan thermocouples, using a Leeds and Northrup portable precision potentiometer.

Farinograph curves were made with some of the sponges and doughs. The sponge curves were made at the end of sponge fermentation, just before the sponge was mixed with the balance of the dough ingredients. The dough curves were made soon after the sponge was mixed with the balance of the dough ingredients. For each curve, 520 g of sponge or dough were used in the farinograph.

Other measurements and observations were made as indicated for the individual experiments. Differences were noted as the doughs went through the makeup machines, and bread score observations were made by several qualified individuals.

Experiment No. 1. In an experiment with the dough room temperature controlled at 80°F (26.7°C) and the sponges mixed at 77°F (25°C), open trough fermentation was compared to cabinet fermentation in 4-hour sponges. At the same time, open and cabinet sponges were fermented for 2 hours and 40 minutes, under similar conditions, to check on the claim that sponge fermentation time in the cabinet may be reduced by one-third.

The results are shown in Table I.

There were no significant differences between open and cabinet fermentation, but the differences between 4 hour and 2 hour and 40 minutes sponge times were definitely significant, under the conditions outlined in Table I (temperature, fermentation loss, and proof

TABLE I

EFFECTS OF VARIATIONS IN SPONGE TIME WITH CABINET AND OPEN TROUGH FERMENTATION

(2% yeast, 60% sponges; dough room, 80°F; sponge, 77°F)

	Final sponge temperature [1]		Sponge ferm. loss	Sponge pH	Sponge consist. after 5½ minutes (farinograph units)	Proof time	Loaf volume
	Center	Edge					
	°F	°F	%			min.	cc
4 hr. sponge; open	89.5	85.0	2.06	4.89	450	69	2,300
4 hr. sponge; cabinet	87.5	85.5	2.06	4.85	430	71	2,290
2 hr. 40 min. sponge; open	85.0	—	1.16	5.00	520	73	2,270
2 hr. 40 min. sponge; cabinet	85.0	—	1.16	5.00	520	74	2,285

[1] Sponge temperature measurements were made by means of long thermometers, both in the center, and at the edge of the sponge in contact with the trough.

time). The cabinet doughs did appear to be slightly more pliable in going through the machines, but the bread showed no difference between open and cabinet fermentation in grain, crumb color, or break and shred. The 4-hour loaves, however, were better developed and showed finer grain and more age than those from the 2 hour and 40 minutes sponge fermentation.

Figure 1 shows the farinograph sponge and dough curves for Experiment No. 1. Significant differences were obtained between the 4 hour and the 2 hour and 40 minutes sponges, as indicated by the rate of breakdown, or loss in consistency during mixing in the farinograph. But there were no differences between the open and cabinet

TABLE II

EFFECTS OF VARIATIONS IN SPONGE TIME WITH CABINET AND OPEN TROUGH FERMENTATION

(1.5% yeast, 66% sponges; dough room, 80°F; sponge, 77°F)

	Final sponge temperature		Sponge ferm. loss	Sponge pH	Sponge consist. after 5½ minutes[1] (farinograph units)	Proof time	Loaf volume
	Center	Edge					
	°F	°F	%			min.	cc
4½ hr. sponge; open	89.75	82.25	2.07	4.90	440	72	2,350
4½ hr. sponge; cabinet	88.75	85.0	2.07	4.92	440	74	2,340
3 hr. sponge; open	84.75	—	1.0	5.14	560	85	2,360
3 hr. sponge; cabinet	84.5	—	1.0	5.16	540	88	2,350

[1] For some of the curves, the farinograph was run longer, but the values given were read at 5½ minutes in order to conform with the values for the 5½ minute curves in Figure 1.

sponges, showing that under our conditions, fermentation in the cabinet produced no measurable changes in water requirements and sponge consistency.

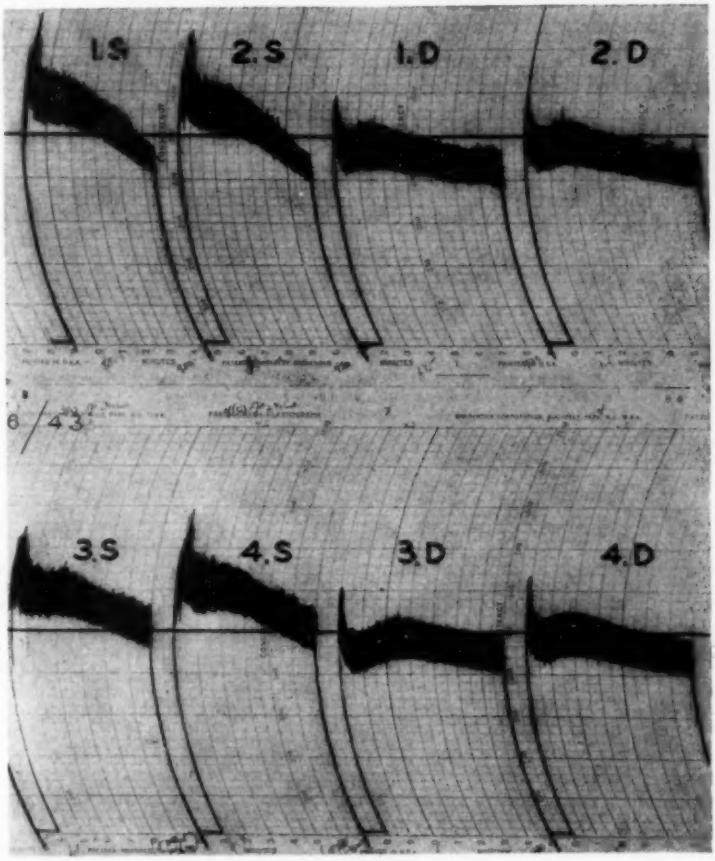

Fig. 1. Effects of variations in sponge time on farinograph curves made from sponges and doughs, after sponge fermentation in cabinets and in open troughs (see Table I).

S = sponge; D = dough
1 = 4-hour sponge; open
2 = 4-hour sponge; cabinet
3 = 2-hour, 40-min. sponge; open
4 = 2-hour, 40-min. sponge; cabinet

The differences between the corresponding dough curves were relatively slight, but again indicated that time of fermentation rather than the use of the cabinet was the important factor here. (The experimental error in these measurements is within 20 farinograph units.)

Experiment No. 2. In this experiment, 66% sponges with 1.5% yeast were used in open trough and cabinet fermentation. The results

are shown in Table II and by the farinograph curves in Figure 2. Here, again, the significant differences were due to time of sponge fermentation, and not to the use of the cabinets.

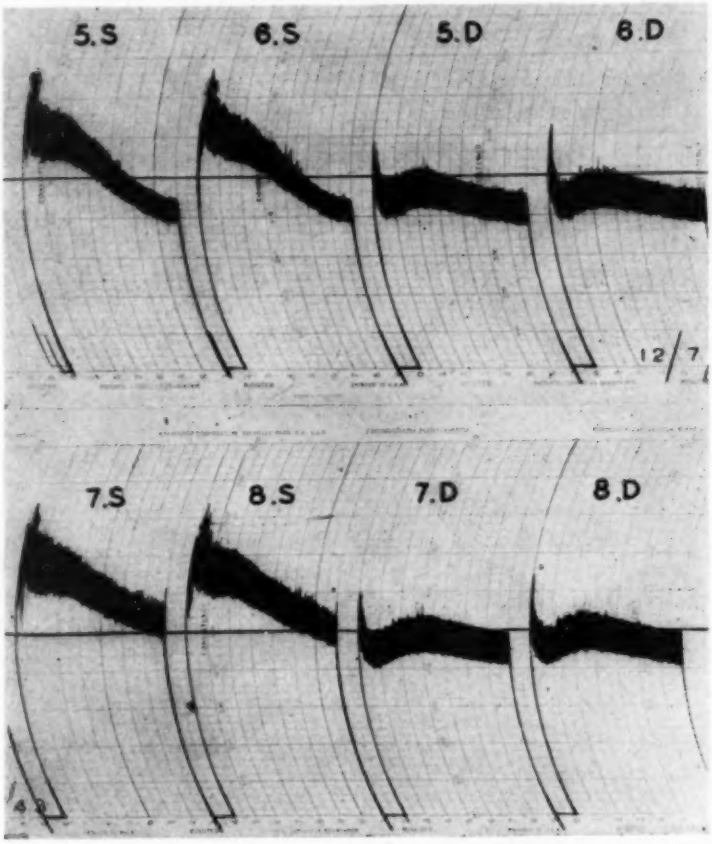

Fig. 2. Effects of variations in sponge time on farinograph curves made from sponges and doughs, after sponge fermentation in cabinets and in open troughs (see Table II).

S = sponge; D = dough
5 = 4½-hour sponge; open
6 = 4½-hour sponge; cabinet
7 = 3-hour sponge; open
8 = 3-hour sponge; cabinet

A slight improvement in handling characteristics was noted in machining the cabinet fermented doughs, but no effects on bread quality could be observed; the loaf resulting from the 3-hour cabinet fermentation was no better than the one from the 3-hour open fermentation, and not as good as the 4½-hour loaves, from either open or cabinet fermentation.

The proof time values are significant; the slow proof due to the

short sponges shows insufficient fermentation, which was not corrected by the use of the cabinet.

The farinograph curves again showed very definite differences due to the time of sponge fermentation, but no significant difference due to sponge fermentation within the cabinets.

Experiment No. 3. In this experiment, the sponges were mixed at 81.5°F, slightly above the dough room temperature. Table III

TABLE III

EFFECTS OF CABINET AND OPEN TROUGH FERMENTATION WHEN USING SPONGES MIXED ABOVE THE NORMAL DOUGH ROOM TEMPERATURE

(2% yeast, 60% sponges; dough room, 80°F; sponge, 81.5°F)

	Final sponge temperature		Sponge ferm. loss	Sponge pH	Sponge consist. at 5½ minutes (farinograph units)	Proof time	Loaf volume
	Center	Edge					
	°F	°F	%			min.	cc
4 hr. sponge; open	93.0	85.75	2.5	4.90	400	69	2,350
4 hr. sponge; cabinet	93.0	85.75	2.5	4.91	410	73	2,375

and Figure 3 show the results obtained. There were no differences between open and cabinet fermentation in this test, either in the sponges, doughs, or the baked bread.

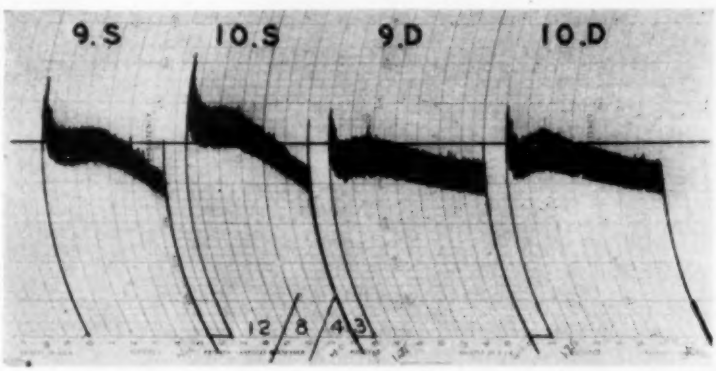

Fig. 3. Farinograph curves made from sponges and doughs after sponge fermentation in cabinets and in open troughs; sponges mixed at temperature above that of the dough room (see Table III).

S = sponge; D = dough
9 = 4-hour sponge; open
10 = 4-hour sponge; cabinet

Experiment No. 4. 60% sponges with 1.5 and 2.0% yeast were used in open and cabinet fermentation, to see whether the lower amount of yeast would show any advantages in the cabinet sponge. Table IV gives the results.

TABLE IV

EFFECTS OF VARIATIONS IN THE AMOUNT OF YEAST WITH CABINET AND OPEN TROUGH FERMENTATION

(4½ hour, 60% sponges; dough room, 80°F; sponge, 77°F)

	Final sponge temperature	Proof time	Loaf volume
	°F	min.	cc
2% yeast; open	90.5	70	2,290
2% yeast; cabinet	90.0	71	2,300
1.5% yeast; open	87.75	80	2,270
1.5% yeast; cabinet	88.5	81	2,255

The differences between open and cabinet fermentation in these tests were negligible, though the cabinet doughs were slightly more mellow in machining. But the differences between 2% and 1.5% yeast were quite distinct in sponge temperature, proof time, and bread score; these were all in favor of 2% yeast, indicating that the use of the cabinet would not permit a reduction in the amount of yeast used.

Experiment No. 5. In this experiment, open trough fermentation in the controlled room at 80°F was compared to open and cabinet fermentation in an uncontrolled room at about 75°F. In another test in the uncontrolled room the top of the trough was covered during the sponge fermentation. The results are shown in Table V.

TABLE V

EFFECTS OF CABINET AND OPEN TROUGH FERMENTATION WHEN USING DIFFERENT DOUGH ROOM CONDITIONS

(2% yeast, 4½ hour, 60% sponges; mixed at 77°F)

	Final sponge temperature	Sponge ferm. loss	Proof time	Loaf volume
	°F	%	min.	cc
Room at 80°F; open	90.0	2.45	69	2,270
Room at 75°F; cabinet	85.0	2.06	74	2,310
Room at 75°F; open	85.5	2.19	74	2,300
Room at 75°F; top covered	85.25	2.06	73	2,290

The differences in sponge temperature, fermentation loss, proof time, and bread score were significant with respect to room temperature, showing that the best fermentation was obtained at 80°F, which also gave the better loaf of bread. Cabinet fermentation at 75°F produced a loaf second in quality, giving better results than open fermentation at 75°F. The covered sponge produced a loaf similar to that of the open trough sponge at 75°F.

It is to be noted that under these uncontrolled conditions at lower room temperature, cabinet fermentation showed some advantage over open trough fermentation in texture and grain, although differences in other respects were negligible.

Use of Thermocouples in Sponge Temperature Measurements

It is reasonable to assume that any benefits derived from cabinet fermentation may be partly due to the insulating effect of the cabinet, thereby minimizing the loss of heat generated during sponge fermentation. In order to get more accurate information on this point, thermocouples were constructed and used for temperature measurement. Figure 4 shows the set-up used for making temperature measurements with thermocouples. Figure 5 shows the positions of the different thermocouples in the trough during sponge fermentation.

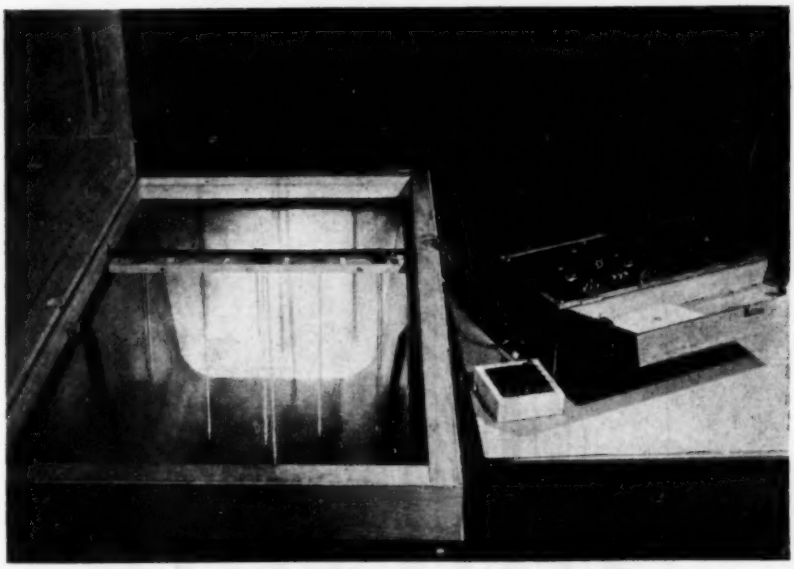

Fig. 4. Set-up used for measuring temperature changes with thermocouples. Dough trough within cabinet, with removable cover at left; thermocouples in glass tubes mounted in strip of wood resting on edges of trough; wires from thermocouples connected through multiple switch (in center) to potentiometer (at right).

Experiment No. 6. Open and cabinet fermentation were compared in regular sponges in the controlled room at 80°F and 96% humidity; the sponges were mixed at 77°F. The results of temperature measurements at different positions in each sponge are illustrated in Figure 6, showing the temperature changes at two points, post 5 in the center of the sponge, and post 7 at the edge of the sponge in contact with the side of the trough.

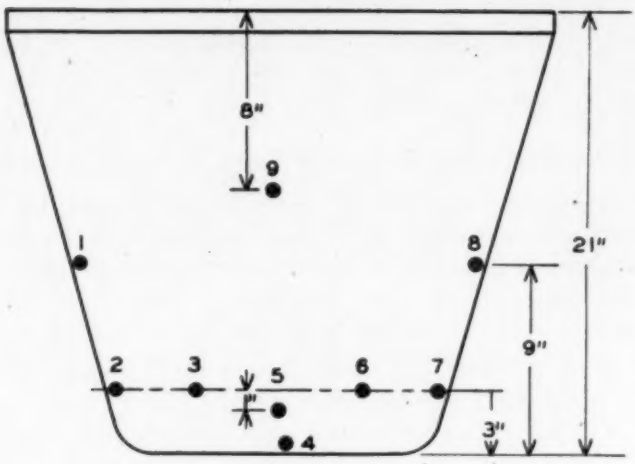

Fig. 5. Positions of thermocouples in dough trough. Each black circle represents a different thermocouple.

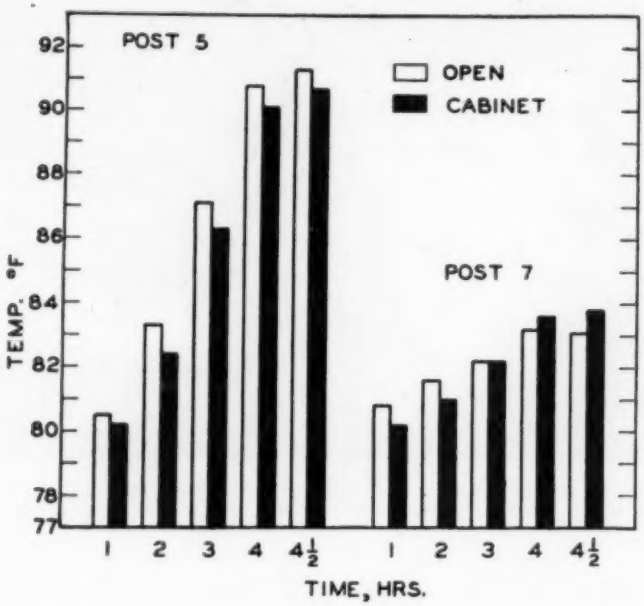

Fig. 6. (Experiment 6.) Temperatures of sponges fermented in cabinets and in open troughs, showing changes in temperature with time, in the center and at the edge of the sponge.

Sponge temperatures
Setting temp. 77°F
Dough room temp. 80°F
Yeast 2%, sponge 60%
Half size sponge with trough dam

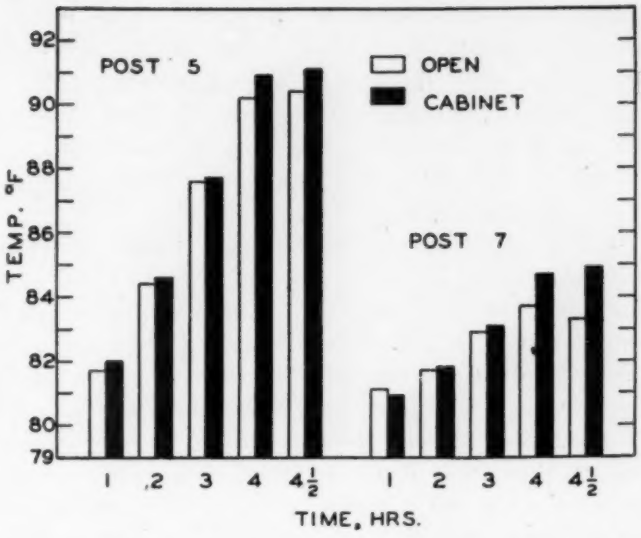

Fig. 7. (Experiment 7.) Temperatures of sponges fermented in cabinets and in open troughs, showing changes in temperature with time, in the center and at the edge of the sponge. Sponges larger than in Experiment No. 6.

Sponge temperatures
Setting temp. 79°F
Dough room temp. 80°F
Yeast 2%, sponge 60%

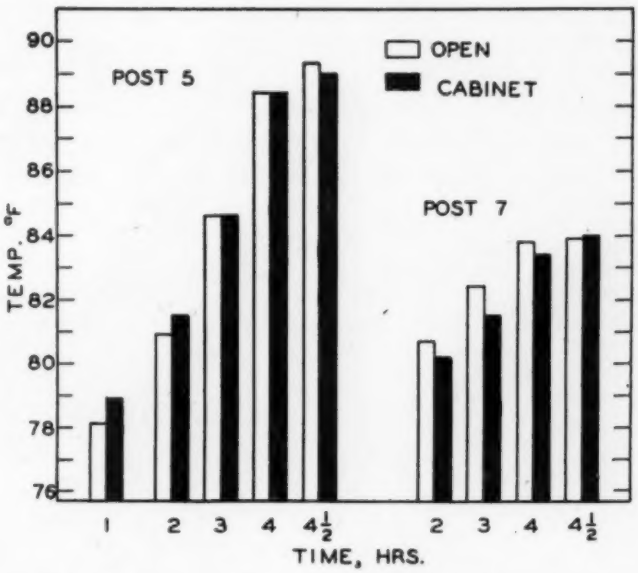

Fig. 8. (Experiment 8.) Temperatures of sponges fermented in cabinets and in open troughs, showing changes in temperature with time, in the center and at the edge of the sponge. Larger sponges with lower initial temperature.

Sponge temperatures
Setting temp. 75.7°F
Dough room temp. 80°F
Yeast 2%, sponge 60%

The temperature differences appear to be insignificant; in the center, the open sponge was about 0.5 degree higher at the end of 4½ hours, while at the edge, the cabinet sponge gained slightly over the open sponge, but was only about 0.5 degree higher after 4½ hours.

The differences in proof time, loaf volume, and bread score were also insignificant, but the cabinet dough was slightly more mellow through the makeup machinery.

Experiment No. 7. In this experiment (Figure 7), double size sponges were used, large enough to fill the trough. The open trough sponge was mixed at 79°F, and the cabinet sponge at 79.5°F; the dough room was at 80°F and 96% humidity. The final temperature in the center was about 0.5 degree higher for the cabinet sponge, but at the edge it was 1.5 degrees higher for the same sponge, partly because of the higher starting temperature. These differences were of little significance, nor were the proof time, volume, and bread score appreciably different. The slightly higher temperature at the edge of the sponge in the cabinet indicates an insulating effect.

Experiment No. 8. This experiment was similar to the preceding one, but the sponge starting temperature was lower; the open trough sponge was mixed at 75.6°F, and the cabinet sponge at 75.7°F.

The temperature readings in this test were almost identical (Figure 8), and differences in fermentation and bread score were negligible; the cabinet dough was slightly more pliable in going through the molder.

Experiment No. 9. Open and cabinet fermentation were now compared in a cool dough room, with the room temperature about 6°F below the starting temperature of the sponge. The results are shown in Table VI.

TABLE VI

EFFECTS OF CABINET AND OPEN TROUGH FERMENTATION IN A COOL DOUGH ROOM
(Dough room 71°F, 96% humidity; 2% yeast, 4 hour, 60% sponges; mixed at 77°F)

	Final sponge temperature		Sponge pH	Sponge consist. at 5½ minutes (farinograph units)	Proof time	Loaf volume
	Center	Edge				
	°F	°F			min.	cc
Open	83.8	73.1	4.89	490	77	2,275
Cabinet	85.4	76.4	4.87	430	79	2,350

In this test, cabinet fermentation was definitely superior in almost all respects. Sponge temperature in the cabinet was higher, both in the center and at the edges, although there was a drop in temperature at the edges in both sponges. The cabinet also produced significant

differences in farinograph sponge consistency, dough handling in the machines, loaf volume, and bread score. Figures 9 and 10 show the sponge temperature and the farinograph curves for this experiment.

The advantages due to the cabinet in this test appear to result mainly from its insulating effect, as indicated by the temperature

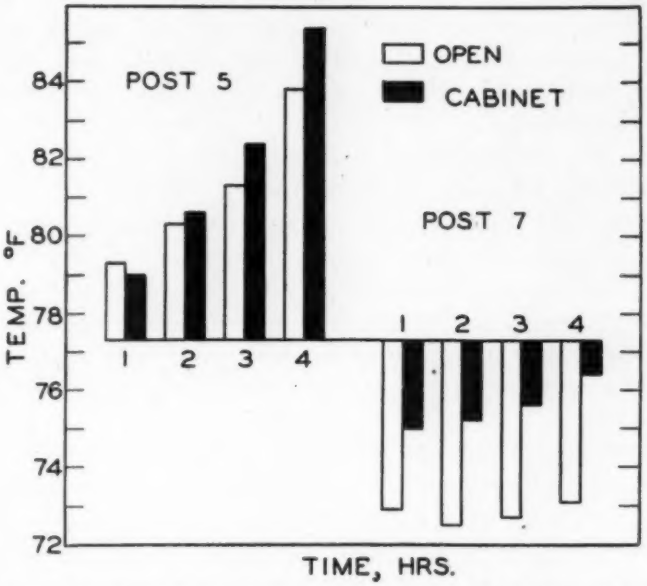

Fig. 9. (Experiment 9.) Temperatures of sponges fermented in cabinets and in open troughs, showing changes in temperature with time, in the center and at the edge of the sponge. Sponges fermented in a cool dough room. Sponge temperature in center lower than in previous experiments; temperature drop at edges.

Sponge temperatures

Setting temp. 77.3°F
Dough room temp. 71°F
Yeast 2%, sponge 60%

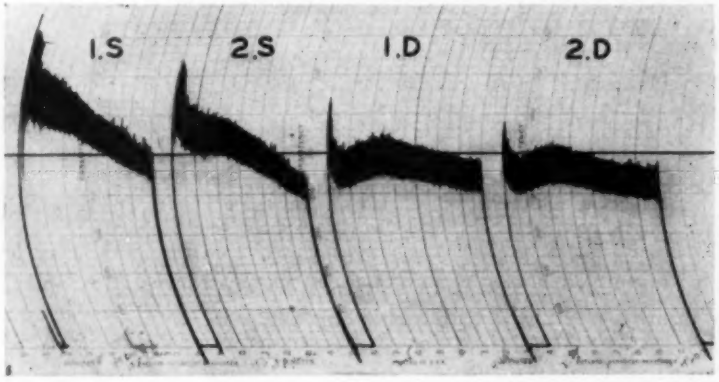

Fig. 10. Farinograph curves made from sponges and doughs after sponge fermentation in cabinets and in open troughs, in a cool dough room.

S] = sponge; D = dough
1] = 4-hour sponge; open
2] = 4-hour sponge; cabinet

measurements; this might have been expected, considering the low temperature of the room in which the sponges were fermented.

Discussion

The work presented in this paper indicates that the effectiveness of cabinet fermentation depends on the conditions under which it is used. Since these may vary, one can make no general statement regarding cabinet fermentation, but must specify and define the conditions used, and indicate in just what respects the results were beneficial or otherwise.

The several measurements and observations used as criteria of effectiveness in this work were convincing. All the data, with the exception of slight mellowing effects, showed cabinet fermentation to offer no advantage over open troughs in a controlled dough room at 80°F.[1] Where cabinet fermentation was found advantageous, as in Experiment 9, the insulating effects of the cabinet in a cool room seem to be primarily responsible, and there is no need to resort to involved explanations, or to ascribe mysterious powers to the cabinet in such instances.

The mellowing effects of cabinet fermentation, as indicated by subjective observations, are yet to be explained, though they may be of minor importance from a practical standpoint, if no other changes are involved. It is unlikely that the "blanket of CO_2" theory is valid, since only a small portion of the total sponge surface is exposed, and for a relatively short time, after the sponge break.[2]

Summary

A comparative study of open trough and cabinet fermentation was made under varying conditions of dough room temperature and humidity, sponge temperature, sponge time, and amount of yeast.

The results indicated that cabinet fermentation was of no value under controlled temperature and humidity conditions in which the room temperature was equal to or greater than the temperature at which the sponge was mixed. But when the dough room temperature was much lower than the starting temperature of the sponge, cabinet fermentation showed definite advantages over open trough fermentation.

It is evident that the effectiveness of cabinet fermentation depends on the conditions under which it is used.

[1] In a few tests with 100% Kansas flour, not reported in this paper, the results were very similar to those obtained with the flour blend used in these experiments.

[2] A few experiments in which carbon dioxide was deliberately admitted into the cabinet above the sponge failed to show any difference not previously observed without its addition.

AMINO ACIDS IN CORN GRAIN FROM SEVERAL SINGLE CROSS HYBRIDS [1, 2]

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It is quite generally recognized that the genetic constitution of a plant may affect its chemical composition, but there is very little published work showing the effect of genetic constitution of the plant on the chemical composition of corn grain. The widespread use of commercial corn hybrids makes it highly desirable to obtain this type of information since the feeding value of corn is dependent, in part at least, on its chemical composition.

The work reported in this paper was conducted to determine whether or not grain from different single crosses among inbred lines of corn varies in its content of protein and certain nutritionally important amino acids. The results indicate that the amounts of tyrosine, tryptophane, cystine, arginine, and histidine present in corn grain are related to the genetic constitution of the plant.

It is known that the protein content of corn grain is variable and may be affected by environmental factors such as soil type, fertilizer treatment, and moisture supply (Doty *et al.*, 1943; Fraps, 1931; Greaves and Nelson, 1925; Whitson *et al.*, 1902; Widstoe, 1903). Data presented in the fifty-first annual report of the Illinois Agricultural Experiment Station (1942) show also that the percentage of protein in corn grain can be increased or decreased by continuous ear-to-row selection.

A few reports have been published on the amino acid composition of the complete protein fraction from corn grain. Csonka (1939) found that Boone County White corn grown in Virginia contained practically the same amounts of cystine, tryptophane, tyrosine, arginine, histidine, and lysine as Black Yellow Dent corn grown in Iowa. Hamilton *et al.* (1921) found that amide nitrogen made up 11.94% of the total nitrogen of corn grain; cystine nitrogen made up 1.07%, arginine nitrogen 8.73%, histidine nitrogen 4.83%, and lysine nitrogen 2.20%. Morris (1934) obtained similar values for flaked maize, except that amide nitrogen and histidine nitrogen were lower.

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[1] Journal Paper No. 221 of the Purdue University Agricultural Experiment Station. Herman Frasch Foundation for Agricultural Research, Paper No. 227.

[2] In cooperation with the Division of Cereal Crops and Diseases, Bureau of Plant Industry, Soils, and Engineering, Agricultural Research Administration, U. S. Department of Agriculture.

[3] Senior Agronomist, U. S. Department of Agriculture.

Zein, a prolamine which makes up 25 to 60% of the total protein of corn grain, has been carefully analyzed for its amino acid content by numerous investigators. This work has been summarized by Hoffman (1925) and Mitchell (1929).

Csonka (1932) and Jones and Csonka (1928) found that α-glutelin from corn contained more lysine, more tryptophane, more arginine, and more histidine than zein.

May and Rose (1922) found that maize gluten contained 1.08% tryptophane, while Matsuyama and Mori (1923) reported that corn protein contained only a trace of tryptophane.

These investigations indicate that the amino acid composition of the complete protein fraction of corn grain may depend upon the relative amounts of the various types of protein present in corn. Osborne and Mendel (1914) found that 22% of the total corn protein was soluble in 10% potassium chloride solution, 41% was soluble in 90% ethanol, 31% was soluble in 0.2% potassium hydroxide solution, and 6% was insoluble or lost. Spitzer, Carr, and Epple (1919) found 5.27% of the nitrogen of corn present as amide nitrogen, 21.61% soluble in 10% sodium chloride solution, 29.41% soluble in 90% ethanol, and 42.81% as glutelin nitrogen. Later, Showalter and Carr (1922) found that greater percentages of the protein of a high-nitrogen corn sample were soluble in 90% ethanol and 10% sodium chloride than of the protein of a low-nitrogen corn sample. More recent work by Zeleny (1935) indicates that the relative proportions of the various proteins in corn grain may be dependent upon the stage of maturity of the corn.

Materials and Methods

The samples of corn grain used in this study were obtained from corn grown on experimental plots on the Soils and Crops Farm near Lafayette, Indiana. In 1939, the samples used were composite samples from triplicate plots. In 1940 and 1941 the samples were taken from single plots. All samples were harvested at maturity, artificially dried, and shelled. The shelled corn was stored at −5°C until ground for analysis.

The methods used for analysis were those described by Doty (1941) with a few exceptions. It was found that in some samples tryptophane could not be determined satisfactorily in an alkali hydrolyzate, hence it was necessary to use the following procedure for the determination of tryptophane.

Place the residue from the saliva digestion (Doty, 1941) of 1 g of dry fat-free corn in a 50 ml beaker. Add 1.0 ml of 2.5% p-dimethylaminobenzaldehyde solution and 0.5 ml of 0.15% sodium nitrate solu-

tion. Stir for 2 minutes, add 25 ml of concentrated hydrochloric acid, and stir. Cover the beaker and allow to stand for 18 hours at room temperature. Dilute the deep blue solution with 10 ml of water, add a pinch of asbestos fiber, and allow to stand for 1 hour at room temperature. Transfer the solution to a 100 ml volumetric flask, add 25 ml of concentrated hydrochloric acid, and make to volume with water. Mix and filter through Whatman No. 42 filter paper. Use a portion of the filtrate to determine the transmission value in the KWSZ photometer. Determine the amount of tryptophane present by reading the transmission value from a standard curve prepared by using known amounts of tryptophane in the procedure outlined above.

It was also found that some batches of Norit A, even after washing with acid and alkali, could not be used for decolorizing the alkaline hydrolyzates because tyrosine was adsorbed to a slight extent. Therefore, the alkaline hydrolyzates were decolorized by shaking about 18 ml of solution in a test tube with approximately 0.3 g of talc and filtering immediately through Whatman No. 42 filter paper.

Histidine determinations on the 1940 and 1941 samples were carried out by the sulfanilic acid method described by Macpherson (1942) except that the color developed was measured in the KWSZ photometer equipped with Corning filter no. 406 and Jena filter GG 11. Several of the samples were analyzed both by this method and the method described by Doty (1941), and it was found that the results agreed perfectly.

Protein fractionation studies were carried out as follows: Extract dry corn, ground to pass an 80 mesh sieve, with dry ethyl ether in a Soxhlet extraction apparatus for 24 hours. Remove the ether from the residue, and shake up 2.5 g of the dry residue with 100 ml of distilled water in a 250 ml centrifuge tube. Let stand for several minutes at room temperature with occasional shaking. Centrifuge, pour off the supernatant liquid, add 75 ml of water, and repeat the extraction. Repeat the water extraction a third time. Make the combined water extracts to volume and use an aliquot for total nitrogen determination. Extract the residue with 5% sodium chloride solution for 1 hour at room temperature with occasional shaking. Centrifuge and repeat the extraction twice. Add 100 ml of 80% ethanol to the residue, mix, and allow to stand overnight. Centrifuge and re-extract twice with 80% ethanol for periods of 1 hour each. Finally extract three times with 0.2% sodium hydroxide solution. Allow the suspension to stand at room temperature for 1 hour with occasional shaking for each extraction. In each case use a total volume of 250 ml of extracting solution, make the combined extracts to volume, and use an aliquot for total nitrogen determination.

In all cases where analysis of variance was made of the data, the standard error of the mean difference was calculated and used to derive the corresponding t value, the significance of which was ascertained from Snedecor's (1938) tables. Throughout the discussion, differences having odds of 19:1 or more against their being due to random variation are termed "significant" and differences having odds of 99:1 or more against their being due to random variation are termed "very significant."

Experimental Results and Discussion

Samples of corn grain from 28 single crosses grown in 1939 were analyzed for total protein, cystine, arginine, histidine, tryptophane, and tyrosine. The results (Table I) show that the protein content of the hybrids varied considerably and that the amino acid distribution of the protein fraction differed with the various samples. There was some indication that protein content and amino acid content of the protein were related to genetic constitution. For example, all hybrids having Mid 1 or Bl 13092 as one parent contained relatively large percentages of protein, while hybrids having 540 or Mo940 as one parent contained relatively small percentages of protein. Also, in 1939, hybrids having Mo940 or 540 as one parent contained less cystine per gram of nitrogen than hybrids of other parentage, while hybrids involving Mid 1 contained relatively large amounts of cystine. Hybrids having Bl 13092 or LE 43 as one parent contained much smaller amounts of arginine than did the remainder of the samples.

The data on the samples grown in 1939 can indicate only trends as regards the influence of genetic constitution on amino acid distribution of the protein, because the effect of only one parent, irrespective of the other parent, can be evaluated. Moreover, the effect of seasonal variation cannot be determined on the basis of one year's results; and seasonal variations are important as shown by the fact that hybrids of the same genetic constitution grown in 1939 and in 1941 varied greatly in total protein and amino acid content (Table I).

To evaluate more closely the effect of genetic constitution on amino acid content, two series of single crosses were grown in 1940 and 1941, and amino acids of the protein fraction from all samples determined. The midseason series consisted of all possible combinations of inbred lines L317, Hy, 38–11, WF9, 187–2, and R4; the late series consisted of all possible combinations of inbred lines Mo940, Hy, 38–11, L317, Mo824, and K4.

The results of the analyses show that there was considerable variation in the amino acid content of the hybrids (Tables II and III). In the first place, season apparently affected the composition to a con-

TABLE I

TOTAL PROTEIN AND AMINO ACID CONTENT OF DRY CORN GRAIN FROM MISCELLANEOUS SINGLE CROSSES

Hybrid pedigree designation	Year grown	Total protein	Amino acid content per gram of nitrogen				
			Cystine	Arginine	Histidine	Trypto-phane	Tyrosine
		%	mg	mg	mg	mg	mg
Mid 1 × YS79	1939	9.4	71	202	132	33.9	372
Mid 1 × 38–11	1939	9.8	81	184	156	31.6	405
Mid 1 × 38–11	1941	10.7	80	215	127	33.6	399
Mid 1 × C9	1939	9.5	69	218	135	33.4	385
Mid 1 × C9	1941	10.5	83	220	133	33.3	394
Mid 1 × KD102	1939	9.9	71	197	147	32.6	387
Mid 1 × Mo824	1939	10.6	79	227	160	31.4	402
Mid 1 × Mo940	1939	8.8	80	234	157	35.0	427
Mid 1 × 540	1939	9.0	71	217	125	31.2	353
Mid 1 × 540	1941	10.0	74	215	124	33.7	394
Mid 1 × K4	1941	10.6	90	212	117	33.6	396
Mo940 × Hy31	1939	8.1	74	186	123	31.3	438
Mo940 × YS79	1939	8.4	56	220	117	34.9	414
Mo940 × 38–11	1939	8.8	52	199	120	35.5	425
Mo940 × C9	1939	7.5	69	189	123	40.5	412
Mo940 × C9	1941	8.9	76	241	120	38.2	434
Mo940 × KD102	1939	7.9	59	185	129	36.5	410
Mo940 × Mo824	1939	8.6	62	182	117	33.5	415
Mo940 × 540	1939	8.4	71	214	138	35.5	398
Mo940 × 540	1941	7.5	77	264	125	45.0	459
540 × Hy31	1939	8.9	55	223	147	33.7	405
540 × Ys79	1939	7.9	55	221	116	35.7	357
540 × C9	1939	7.9	72	183	153	39.5	400
540 × C9	1941	8.1	79	260	132	44.2	428
540 × 38–11	1939	8.9	68	216	130	31.8	405
540 × 38–11	1941	8.9	69	253	133	40.5	410
540 × KD102	1939	8.0	68	200	137	36.5	401
540 × Mo824	1939	8.4	65	211	131	35.5	434
540 × K4	1941	9.3	83	253	129	40.7	444
LE43 × 38–11	1939	9.1	78	132	140	30.7	380
LE43 × WF9	1939	8.4	74	144	118	31.8	367
LE43 × 540	1939	8.6	58	140	156	32.7	385
LE43 × P8	1939	9.6	66	135	160	29.0	351
Bl 13092 × R4	1939	9.8	63	131	152	29.6	367
Bl 13092 × R4	1941	9.4	81	239	131	38.2	388
Bl 13092 × 38–11	1939	10.2	76	150	142	29.4	375
Bl 13092 × 540	1939	9.8	51	139	129	29.9	360
Bl 13092 × 540	1941	10.4	81	215	131	32.6	420
Bl 13092 × Hy	1941	10.2	76	231	130	36.3	411
Bl 13092 × WF9	1941	11.9	88	233	146	33.5	367
C9 × K4	1941	10.5	78	230	123	36.8	398
C9 × 38–11	1941	9.7	78	223	120	36.7	407
C9 × Mo824	1941	10.0	74	230	121	36.9	403

All figures given are averages from at least two separate determinations which did not vary from the average by more than 5%.

TABLE II

TOTAL PROTEIN AND AMINO ACID CONTENT OF DRY CORN GRAIN FROM A SERIES OF MIDSEASON SINGLE CROSSES

Hybrid pedigree designation	Year grown	Total protein	Amino acid content per gram of nitrogen				
			Cystine	Arginine	Histidine	Trypto-phane	Tyrosine
		%	*mg*	*mg*	*mg*	*mg*	*mg*
L317 × Hy	1940	9.6	81	208	109	31.2	396
L317 × Hy	1941	9.8	77	210	123	33.0	384
L317 × 38–11	1940	10.4	78	217	113	26.5	374
L317 × 38–11	1941	10.5	79	202	126	30.9	373
L317 × WF9	1940	10.5	79	208	125	30.4	369
L317 × WF9	1941	8.5	81	243	135	36.0	378
L317 × 187–2	1940	10.4	74	194	112	30.0	368
L317 × 187–2	1941	9.4	81	258	127	34.6	361
L317 × R4	1940	9.4	75	211	105	32.7	387
L317 × R4	1941	9.0	79	228	127	30.0	358
Hy × 38–11	1940	9.8	74	218	120	28.7	427
Hy × 38–11	1941	9.8	73	223	119	33.0	394
Hy × WF9	1940	9.8	63	153	111	31.8	395
Hy × WF9	1941	9.1	88	234	128	36.5	377
Hy × 187–2	1940	9.3	74	215	125	33.5	428
Hy × 187–2	1941	9.5	79	230	124	33.6	395
Hy × R4	1940	9.2	76	218	105	36.3	422
Hy × R4	1941	9.8	80	223	123	35.6	384
38–11 × WF9	1940	10.9	76	200	125	26.3	377
38–11 × WF9	1941	10.4	83	217	126	32.6	366
38–11 × 187–2	1940	10.5	67	196	110	25.6	357
38–11 × 187–2	1941	10.0	74	206	117	31.8	385
38–11 × R4	1940	10.5	77	190	112	29.8	405
38–11 × R4	1941	11.0	80	250	124	31.8	379
WF9 × 187–2	1940	10.1	75	210	131	31.4	389
WF9 × 187–2	1941	10.4	83	223	132	36.8	356
WF9 × R4	1940	10.1	73	197	113	29.8	370
WF9 × R4	1941	9.3	83	235	130	35.5	351
187–2 × R4	1940	9.2	80	218	114	32.6	394
187–2 × R4	1941	9.9	75	221	133	35.5	367

See footnote, Table I.

siderable extent. Analysis of variance of the data showed that for the series of midseason hybrids, the average values for cystine, arginine, histidine, and tryptophane were all very significantly higher in 1941 than they were in 1940. For the late series, the average histidine

TABLE III

TOTAL PROTEIN AND AMINO ACID CONTENT OF DRY CORN GRAIN FROM A SERIES OF LATE YELLOW SINGLE CROSSES

Hybrid pedigree designation	Year grown	Total protein	Amino acid content per gram of nitrogen				
			Cystine	Arginine	Histidine	Trypto-phane	Tyrosine
		%	*mg*	*mg*	*mg*	*mg*	*mg*
Mo940 × Hy	1940	9.6	70	210	119	29.9	396
Mo940 × Hy	1941	11.4	74	229	124	31.2	393
Mo940 × 38–11	1940	9.9	78	206	122	29.7	393
Mo940 × 38–11	1941	11.4	76	218	124	31.6	383
Mo940 × L317	1940	8.9	88	211	111	30.3	444
Mo940 × L317	1941	10.0	83	238	138	32.5	381
Mo940 × Mo824	1940	10.5	66	179	102	26.7	357
Mo940 × Mo824	1941	11.0	72	221	116	31.3	379
Mo940 × K4	1940	10.1	75	192	109	29.6	395
Mo940 × K4	1941	11.1	78	236	121	33.1	378
Hy × 38–11	1940	9.7	72	213	113	29.1	393
Hy × 38–11	1941	10.3	70	224	112	33.3	398
Hy × L317	1940	8.5	77	206	107	33.8	427
Hy × L317	1941	9.3	76	222	110	34.2	398
Hy × Mo824	1940	9.9	70	197	102	28.4	367
Hy × Mo824	1941	10.0	75	225	111	33.7	400
Hy × K4	1940	10.2	74	190	101	28.2	410
Hy × K4	1941	10.8	73	220	109	32.4	401
38–11 × L317	1940	9.4	83	220	142	29.7	391
38–11 × L317	1941	8.7	82	230	129	35.2	425
38–11 × Mo824	1940	10.2	72	190	107	26.7	368
38–11 × Mo824	1941	10.3	78	218	115	31.5	404
38–11 × K4	1940	10.2	80	208	116	26.4	393
38–11 × K4	1941	10.0	85	231	134	35.0	437
L317 × Mo824	1940	10.1	80	179	110	30.2	383
L317 × Mo824	1941	10.4	77	217	116	31.9	416
L317 × K4	1940	10.0	81	200	107	29.3	405
L317 × K4	1941	8.1	78	238	121	36.1	430
Mo824 × K4	1940	11.1	69	168	90	26.4	366
Mo824 × K4	1941	8.7	83	236	129	35.3	425

See footnote, Table I.

content was significantly higher, and the average values for arginine and tryptophane were very significantly higher in 1941 than in 1940.

From inspection of the data in Tables II and III it appears that in some cases the amino acid content of the protein of the hybrid was

related to the genetic constitution. For example, among the midseason crosses, all hybrids involving 38–11 were somewhat lower in tryptophane than were hybrids with other parentage. In order to determine whether or not such differences were significant, the data from each series were separated into groups of hybrids with one common parent and the data for each group compared to that for a second group with a different common parent. For example, the group consisting of L317 × 38–11, L317 × WF9, L317 × 187–2, and L317 × R4 was compared with the group consisting of Hy × 38–11, Hy × WF9, Hy × 187–2, and Hy × R4. In this way the relative effects of L317 and Hy on the amino acid content of the hybrids could be ascertained.

TABLE IV

SIGNIFICANT DIFFERENCES IN THE PROTEIN AND AMINO ACID CONTENT OF GRAIN FROM MIDSEASON CROSSES AS RELATED TO THE INBREDS INVOLVED

Protein	Hybrids involving 38–11 higher than L317, Hy,* 187–2,* or R4* crosses
Tryptophane	Hybrids involving Hy higher than L317,* WF9,* 187–2,* R4,* or 38–11 crosses
Tyrosine	Hybrids involving Hy,* WF9,* 187–2,* or R4* higher than 38–11 crosses
Histidine	Hybrids involving WF9 higher than L317, Hy, 38–11, or R4 crosses Hybrids involving 187–2 higher than Hy crosses
Cystine	No significant differences
Arginine	No significant differences

* Very significant (>99:1).

TABLE V

SIGNIFICANT DIFFERENCES IN THE PROTEIN AND AMINO ACID CONTENT OF GRAIN FROM LATE YELLOW SINGLE CROSSES AS RELATED TO THE INBREDS INVOLVED

Protein	Hybrids involving Hy, Mo940,* 38–11, Mo824,* or K4 higher than L317 crosses
Tryptophane	Hybrids involving L317 higher than Mo940 or Hy crosses
Tyrosine	Hybrids involving L317 higher than Mo940* or Mo824 crosses Hybrids involving K4 higher than Mo824 crosses
Cystine	Hybrids involving L317 higher than Mo940,* Mo824,* or Hy* crosses Hybrids involving K4 higher than Hy crosses Hybrids involving 38–11 higher than Hy* crosses
Arginine	Hybrids involving K4,* 38–11, Mo940, or Hy higher than Mo824 crosses
Histidine	Hybrids involving 38–11 higher than Hy crosses Hybrids involving Mo940 higher than Mo824 crosses

* Very significant (>99:1).

Analysis of variance on data organized in this way showed that in many cases the amino acid content of the hybrids was significantly affected by the parentage of the hybrids (Tables IV and V). These effects were actually on the protein and were not necessarily related to the total protein content of the corn grain, since the amino acid contents were expressed as the amounts of amino acids per unit of nitrogen.

The data and the discussion just completed indicate that the amino acid distribution in corn protein is heritable, and may be controlled, to some extent at least, by choosing the inbreds which are used to develop a hybrid. Since the amino acid distribution in the protein was shown to be different in different hybrids, then the physico-chemical behavior of grain protein from different hybrids might be expected to vary. To test this, the solubility of the nitrogen in the grain from two hybrids grown in 1940 was determined. The results (Table VI) show that the solubility characteristics of the protein from

TABLE VI

AMINO ACID COMPOSITION AND PROTEIN-SOLUBILITY OF GRAIN FROM TWO SINGLE CROSS HYBRIDS GROWN IN 1940

	L317 × 38–11	Mo940 × Mo824
Total protein (% on dry wt. basis)	9.4	10.5
Cystine (mg per g N)	83	66
Arginine (mg per g N)	220	179
Histidine (mg per g N)	142	102
Tryptophane (mg per g N)	29.7	26.7
Tyrosine (mg per g N)	391	357
Nitrogen sol. in H_2O (% of total N)	10.8	12.6
Nitrogen sol. in 5% NaCl (% of total N)	12.0	12.8
Nitrogen sol. in 80% C_2H_5OH (% of total N)	20.5	23.3
Nitrogen sol. in 0.2% NaOH (% of total N)	48.4	43.4
Nitrogen of insoluble residue (% of total N)	12.7	10.5
Nitrogen recovered (% of total N)	104.4	102.6

the two samples were distinctly different. The grain from L317 × 38–11 contained more alkali-soluble and less alcohol-soluble nitrogen than did grain from Mo940 × Mo824. This is in harmony with the fact that L317 × 38–11 contained greater proportions of cystine, arginine, histidine, tryptophane, and tyrosine in its protein than did Mo940 × Mo824, since reports by Csonka (1932), Hoffman (1925), Jones and Csonka (1928), May and Rose (1922), and Mitchell (1929) show that the glutelin (dilute alkali-soluble) fraction of corn protein contains greater proportions of these amino acids than does the zein (alcohol-soluble) fraction. If the view of seed proteins as elaborated by Gortner (1938) is accepted, then the results discussed above must

be interpreted to mean that the protein complex of the two hybrids was distinctly different.

In general, the amino acid content of corn grain as reported here agrees very well with results published by Csonka (1939), Hamilton *et al.* (1921), and Morris (1934).

To our knowledge, it has been shown for the first time that the amounts of certain amino acids in corn protein are affected, to some extent at least, by the genetic constitution of the plant. The occurrence of several highly significant differences in amino acid content among single crosses from a few random inbred lines indicates that in all probability a wider range in composition could be found if a larger number of inbred lines were studied similarly. Therefore, it might be possible by careful breeding to develop a hybrid with a high proportion of nutritionally essential amino acids in its protein.

Summary

The grain from 28 corn single crosses grown in 1939 was analyzed for total protein, tryptophane, tyrosine, cystine, arginine, and histidine. There was some indication that the amount of the various amino acids in the corn protein was related to the genetic constitution of the hybrids.

In many cases the amino acid content of the corn protein was related to the inbred lines involved in single cross hybrids grown in 1940 and 1941.

The physico-chemical nature of the protein in the grain from two single cross hybrids was distinctly different as shown by the fact that the sample which contained the larger amounts of cystine, arginine, histidine, tryptophane, and tyrosine also contained a larger percentage of alkali-soluble nitrogen and a smaller percentage of alcohol-soluble nitrogen.

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AN AUTOMATIC DEVICE FOR THE MEASUREMENT OF GAS PRODUCTION AND GAS RETENTION IN DOUGHS [1]

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Numerous published papers dealing with the measurement and significance of gas production and gas retention in fermenting doughs have been reviewed by Elion (1940). The gas produced may be measured volumetrically, as by the procedure of Bailey and Johnson (1924). Similar devices have been described by Jørgensen (1931), Markley and Bailey (1932), Heald (1932), Sherwood, Hildebrand, and McClellan (1940), and Hullett (1941). Gas production has been measured manometrically by Blish, Sandstedt, and Astleford (1932) and by the widely used apparatus of Sandstedt and Blish (1934) and its modification (Malloch, 1939). An automatic, manometric apparatus has been described by Miller, Edgar, and Whiteside (1943). A gravimetric procedure has been described by Elion (1939). Eva, Geddes, and Frisell (1937) compared several methods of measuring gassing power and found the correlation between them to be of very high degree.

The apparatus described in this paper embodies the principles used in the Chefaro balance (Elion, 1939) except that, instead of using weight as a method of measurement, the pressure of the gas produced during fermentation in a sealed jar of known and constant volume is automatically recorded by electric sparks which burn holes in a chart paper. Many of the mechanical principles used were suggested by Mr. C. F. Buck who constructed the apparatus used by Heald (1932).

Description of Apparatus

The apparatus, illustrated in Figure 1, consists principally of a constant temperature water bath, two revolving aluminum drums holding the graph paper on which the data are recorded, four mercury manometers which activate a stylus, glass jars weighed down so as to remain under water during the course of the experiment, and an electrical circuit for producing the sparks.

The water bath, size 18″ × 12″ × 7″, is large enough to hold four jars, a mechanical stirrer to keep the water agitated, a thermometer,

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and a thermoregulator. The temperature of the bath is maintained at 30°C during measurements.

The jars are wide-mouth, 4 inches high, and $3\frac{3}{4}$ inches in diameter outside measurement, and each of 500 ml volume. Inside each jar is

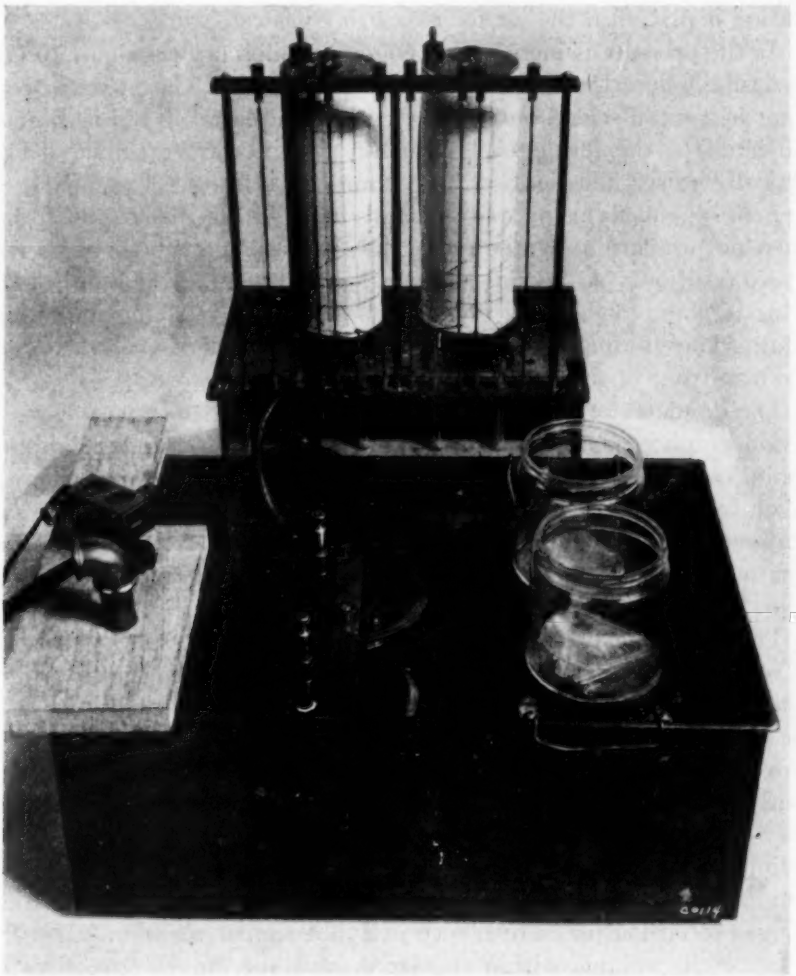

Fig. 1. Original automatic recording gasometer at rest.

placed a glass triangle (bent glass tubing) to support a watch glass upon which the dough is placed. During operation, the jars are sealed gastight with rubber-gasketed brass lids which are bolted to a brass platform. This can be raised when placing and removing doughs. On each brass lid is a release valve and also an outlet which is coupled with a mercury manometer by means of thick-walled rubber

tubing. The pressure in the jars is produced by the expansion of the fermenting dough and also by the carbon dioxide which escapes from the dough, unless the latter is absorbed by alkali. A 23% sodium chloride solution (25 ml) is used for measuring gas production and a 23% potassium hydroxide (or other alkali such as sodium hydroxide) solution is placed in the jar for measuring gas retention.

As the pressure is increased, a column of mercury rises in a 10 mm glass tube whose lower end is below the surface of the mercury container in a small sealed bottle which acts as a reservoir and is directly connected to the fermentation jars by heavy rubber tubing. This vertically raises the floating aluminum-cork stylus. The height of the point of the stylus measures the pressure within the connected jar. When no pressure activates the stylus, the recording point is at rest at zero position. At all times, regardless of pressure, the point of the stylus is about one-eighth of an inch from the revolving aluminum drum. The guiding of this point is by means of the vertical rods shown in front of the drums.

The diameter of the revolving drums is such that the usual cross-section graph paper (cross-ruled in 1/20 of an inch) can be wrapped around once. Each drum is rotated by a clockwork gear mechanism driven by an electric clock motor of one rpm. Each drum has a circumferential speed of one inch per hour and will rotate for 8 hours and then automatically shut off. Any shorter period can be used by properly adjusting the drums.

The recording of the pressures in the jars is by sparks from an A.C.-operated induction coil. The duration of sparking is controlled by adjusting the position of a contact switch on the clock motor shaft. The circuit for a particular stylus closes and a spark passes from the point of the stylus across the gap and into the aluminum drum. This burns a small hole through the graph paper. Each stylus sparks once every 4 minutes, leaving 15 dots per inch of horizontal travel, thus outlining a continuous graph of the pressure within the connected jar from the start of operation until the machine automatically stops. The gas retention pressure is recorded in a similar manner.

Since the volume within the jar is constant, any increase in the internal pressure is directly proportional to the volume of the gas produced (or retained). The volume of gas produced or retained is therefore determined from the pressure readings on the graph by means of an equation based on Boyle's law. For the particular volume of the jars used, an increase in pressure of one inch of mercury equals 15.88 ml of gas.

The apparatus has a capacity of four samples, the dough having the same consistency as that used for bread. Four samples may be

run for gas production only or gas retention only, or two samples for gas production and two for gas retention, depending on whether a solution of sodium chloride or of sodium hydroxide is placed under the watch glasses.

A pair of typical curves is given in Figure 2, the upper showing total production and the lower total retention. The pressure is measured on the Y-axis and the time of fermentation on the X-axis.

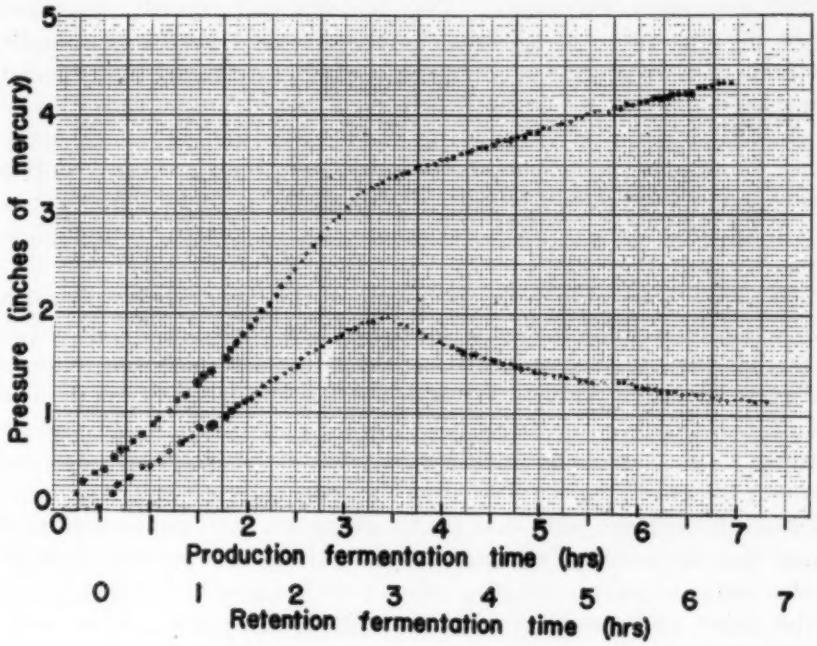

Fig. 2. Typical gas production (top) and gas retention curves obtained from automatic recording gasometer.

Both the production and retention curves are started at the same time; but because of the arrangement of the stylus, the production curve lags behind the retention curve. Correct comparison of the two curves requires adjustments for the starting points, with concomitant changes in the relative positions of the remainder of the curves.

The change in slope of the production curves is the point at which the reserve supply of fermentable sugars reaches a critically low level in so far as the rate of fermentation is concerned. The peak of the retention curve is at the point of maximum gas retention. Although the height of the curves at any point on the chart is directly measurable in inches, a simple calculation (15.88 $\times$ height in inches) transforms the height to milliliters of gas.

Experimental

The gassing power of winter, spring, and soft wheat flours was determined by both the Sandstedt and Blish (1934) manometric apparatus and the automatic recording apparatus described above on six consecutive days. The procedure described by Sandstedt and Blish (1934) was followed for the manometric method. The method is described in *Cereal Laboratory Methods* (4th ed., 1941). Only flour, water, and yeast were used. Two and one-half percent yeast and optimum absorption and mixing time were used for the automatic method. A 10-g aliquot of the dough, which was mixed by a regular mixograph apparatus, was used with the automatic apparatus.

In the comparative study of the manometric and automatic apparatuses only gas production comparisons were made. Gas retention measurements are possible and practical on the automatic apparatus, and frequently for gas retention studies regular bread formulae are used.

Readings were taken from the gauges of the manometric apparatus every half hour for a period of 6 hours (12 readings). As an aid in obtaining the pressure values from the automatic recording data, a flat glass on which parallel, vertical lines had been ruled was used to obtain the values recorded on the graph paper of the automatic recording gasometer. The lines were spaced at intervals equal to the distance the graph paper traveled on the rotating drum in half an hour. By placing the glass plate over the graph paper, with the first vertical line at the point of zero fermentation time, the succeeding intersections of the vertical lines on the glass plate with the graph recorded on the graph paper gave readings from the automatic recorder which were comparable with the readings from the manometric apparatus. It is these readings which have been used in comparing the two methods.

Results and Discussion

Table I gives the average values of the six replications, according to the type of flour, apparatus, and fermentation time. The manometric readings are in terms of gauge pressure. The automatic readings are in terms of units of mercury. Inasmuch as the graph paper used on the automatic apparatus was ruled 20 lines to the inch a unit of mercury pressure has been taken as 0.05 inch of mercury.

The linear relationship between the manometric and automatic readings was $Y = 4.58x$, where Y = manometric readings and x = automatic readings. The correlation between the data was + .978 which was of very high degree as well as highly significant. Only 4.4% of the variation was found to be independent of the correlation. The

TABLE I

MEAN VALUES OF READINGS FROM THE MANOMETRIC AND AUTOMATIC GASOMETERS

Reading No.	Fermentation time	Flour types						Experimental average	
		Soft		Spring		Winter			
		Method							
		Manometric apparatus[1]	Automatic apparatus[2]	Manometric apparatus	Automatic apparatus	Manometric apparatus	Automatic apparatus	Manometric apparatus	Automatic apparatus
	hrs.	*mm Hg*		*mm Hg*		*mm Hg*		*mm Hg*	
1	0.5	52	12	51	11	42	10	49	11
2	1.0	104	23	103	21	93	21	100	22
3	1.5	156	33	158	34	150	31	155	33
4	2.0	206	44	235	48	218	45	230	46
5	2.5	241	53	298	64	286	59	275	59
6	3.0	257	58	367	78	348	74	324	70
7	3.5	272	61	426	91	400	87	366	80
8	4.0	282	63	464	103	432	96	393	87
9	4.5	291	66	495	109	459	102	415	92
10	5.0	301	68	517	113	478	105	432	95
11	5.5	313	71	538	116	491	108	447	98
12	6.0	319	73	555	119	509	112	461	101

[1] Gauge pressure.
[2] Units of mercury (20 units = 1 inch mercury). One unit of mercury = 1.29 mm.

correlations between the two methods, according to flour type, were + .959, + .983, + .983 for spring, winter, and soft wheat flour, respectively. The change in rate of gas production occurred at the same time with both methods.

The standard error, stated in terms of percentage of the respective means, of the manometric apparatus was 10.9% and that for the automatic apparatus was 6.8%.

The data thus indicate that the same results would be obtained with the automatic apparatus as with the regular A.A.C.C. method originally described by Sandstedt and Blish (1934). The automatic apparatus has the added advantages that it does not require the attention of an operator from the time it is started until it automatically shuts itself off and a new sample can be tested, and data are obtained with appreciably greater accuracy than with the regular method.

Summary

A new type automatic recording gasometer has been described. It was compared with the regular A.A.C.C. manometric method of determining gassing power.

The correlation between the two methods was of very high *degree* as well as highly significant (.978).

The standard errors were 10.9% and 6.8% for the regular manometric and automatic methods, respectively.

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EFFECT OF FLOUR LIPIDS ON RECOVERY OF GLUTEN FROM HARD AND SOFT WHEAT FLOURS BY THE USE OF SULFITE SOLUTIONS [1]

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The existence of a connection between flour lipids and some properties of flour is generally admitted, but the connection itself remains obscure. Most experimenters seem agreed that the extraction of lipids improves the properties of the bread; but there is less agreement on the effect produced by reincorporation of the extracted material.

Working (1924) increased the tenacity of poor gluten by washing phosphatides therefrom, and observed that the addition of phosphatide to normal flour injured the gluten quality. Similarly, Johnson and Whitcomb (1931) found that doughs (and breads) made from ether-extracted flour were superior to those from the originals; but the addition of lard to doughs made from the extracted flours markedly reduced their retention of gas. On the other hand, Sullivan, Near, and Foley (1936), while observing that the extraction of flour with ether did not reduce the loaf-volume or the quality of the dough, found that readdition of wheat lipids or fractions thereof also had no great effect on these properties (unless the lipids had become oxidized). Fisher and Jones (1936) found an improvement in flour owing to the removal of lipids, and a still further improvement when they were put back. Sinclair and McCalla (1937) have pointed out the importance of lipids to the characteristics of flours, and the experiments which they report show the complexity of the phenomena involved.

Changes in the lipid content of a flour are obviously reflected in the composition of the isolated crude gluten, because of the large quantities of lipids which the latter carries. Gluten prepared by Fisher and Halton (1933) contained about three-fourths of the total flour lipids.[2] Furthermore, Sullivan and Near (1927) observed the lipid content to be nearly the same in widely different wheats, and called attention to the fact that therefore the ratio of lipid to protein is much greater in soft than in hard wheat. Moreover, Baker and Mize (1942) found that the effect of added lipids on the baking qualities of natural flours depends more on the physical properties than on the

[1] Enzyme Research Laboratory Contribution No. 98. Part of this work was done under Special Research Fund authorized by the Bankhead-Jones Act of June 29, 1935.

[2] Samples of sulfite gluten prepared in this laboratory from hard wheat flour and analyzed by Dr. W. G. Rose contained 6 to 9% of lipids, thus accounting for roughly two-thirds of the total in the flour. The glutens were hydrolyzed by acid or by papain before exhaustive extraction, and the extract was subsequently purified.

chemical composition of the fatty material. While solid fats in general improved the texture and crumb more than did liquid fats, the composition of the solid was of minor importance, for certain waxes and hydrogenated true fats gave equally good results. It appears then that the effect of adding lipids to flour depends to a small extent upon the fatty material added, but to a large extent upon the type of flour used, i.e., hard or soft wheat.

Experimental

A curious difference observed in this laboratory between hard and soft wheats is in the proportion of the total protein that can be recovered as a curd after agitating the flour with air and warm dilute sulfite solution according to the method of Balls and Tucker (1943). From hard wheat flours much of the protein can be recovered in this manner and washed until practically free from starch. With flour from soft wheats, however, similar treatment has resulted in low recoveries of protein, partly because less curd was formed and also because the curd did not cohere well enough to be washed thoroughly. It was therefore of interest to learn if a reduction in the lipid content of flour would lead to a better coagulation of the protein upon treatment with sulfite. This was found to be the case with soft wheat flour, but not with flour from hard wheat. The baking properties of the flour used are outside the scope of these experiments. There seems to be no obvious connection between the yield of protein recovered in this manner and the behavior of the flour in baking, other than the difference always implied between hard wheat and soft wheat flours.

In the experiments reported here, three unbleached patent flours were used; one was from hard and two from soft wheat. The hard wheat flour (A) was made from a winter wheat and contained 15.0% protein. The soft wheat flours were (B) a cracker flour, containing 10.4% protein, made from a western white wheat, and (C) a cookie flour, containing 6.5% protein, also made from a western white wheat.

Large quantities of each flour were extracted with low-boiling petroleum ether in glass percolators until the effluents were completely colorless.[3] As much solvent as possible was then removed by suction and the remainder was allowed to evaporate in a current of air without heating. The extracted lipids were concentrated by removing a large portion of the solvent on a steam bath. When it was desired to reincorporate some of the extracted lipids into the flour, the latter was mixed with a solution of the lipids in petroleum ether, and the solvent was allowed to evaporate spontaneously while the mixture was stirred

[3] The extraction was assumed not to have removed the bound lipids; this is not strictly true, because substances resembling protamines have been found in the petroleum ether extract. As far as is known, however, their amount is relatively small.

in a current of air. In this way any desired proportion of the extract could be added, with good distribution, to the extracted flour.

The amount of extractable lipids was determined analytically in a Soxhlet apparatus, ethyl ether being used instead of petroleum ether, since the two solvents remove practically the same fat fraction. The lipid material remaining in the large batches of flour after extraction by petroleum ether was determined analytically by washing out the lipids with ethyl ether after the lipid-protein complexes had been broken down by long exposure to a mixture of ether and absolute alcohol. In detail, 100 g of previously extracted flour was shaken frequently for 48 hours at room temperature with 400 ml of a mixture of equal volumes of absolute alcohol and ether. The mixed solvents were filtered off, and the flour washed with ether. The combined liquids were evaporated directly to dryness, and the residue weighed. The results are given in Table I. The reason for accepting values

TABLE I

LIPID MATERIAL EXTRACTED FROM THREE FLOURS BY ETHYL ETHER AND THEREAFTER BY ETHER AND ALCOHOL

Flour	Protein in flour	Lipids extracted by ethyl ether	Lipids subsequently extracted by alcohol and ether	Total lipid
	%	%	%	%
A	15.0	0.99	0.31 [1]	1.30
B	10.4	1.00	0.30 [1]	1.30
C	6.5	1.10	0.33 [1]	1.43

[1] In the case of flour A, this fraction contained 0.92% of phosphorus (equivalent to 23% lecithin) and 0.92% of sterols. In the case of flour B, it contained 0.73% of phosphorus (equivalent to 18% lecithin) and 1.2% of sterols. Flour C contained 0.97% of sterol. Phosphorus was determined by the method of Fiske and Subbarow (1925); sterols by the method of Pijoan and Walter (1937).

obtained in this manner was that two other schemes of analysis by which the protein-lipid complexes were more obviously decomposed gave substantially the same results.[4] It is suggested that no present method for the determination of "bound lipids" in gluten is entirely beyond criticism; however, there is evidence that in the results presented here the easily extractable lipids play the larger role.

The protein was coagulated by putting 8 liters of water containing 8 g of a mixture of sodium sulfite and sodium acid sulfite (in the pro-

[4] A 12.5-g portion of flour A was digested with 0.13 g of a very active papain paste in 50 ml of water containing 1 ml of 2 *M* KCN for 18 hours at room temperature. The digest was then diluted with 300 ml of a 1:1 mixture of normal alcoholic HCl and ether. After 5 hours the mixture was poured into 1.5 liters of water. The ether layer was removed. The aqueous layer was extracted twice more with suitable quantities of ether, and the combined ether extracts were then washed thrice with water. The ether was dried over sodium sulfate and evaporated. The residue was weighed. By this method 0.29% of the previously extracted flour was recovered as lipids.

In another experiment a 25-g portion of flour A was treated for 48 hours at room temperature with a mixture of 150 ml *N* HCl in absolute alcohol and 150 ml ether. The flour was filtered off and washed well with ether. The combined liquids were evaporated to about 25 ml and then poured into about 500 ml of water. The watery emulsion was repeatedly extracted with ether; the ether was dried and evaporated. The residue, amounting to 0.35% of the flour, was recovered. From flour B, 0.32% was obtained by the same procedure.

portion to give a pH of 6.0, when dissolved) into a 15-gallon barrel churn, and then adding 2400 g of the flour. The churn was rotated for 30 minutes at 45 rpm. The temperature of the contents fell from 40° to 35°C. After churning, the coagulated protein was permitted to rise to the top, washed thoroughly with cold water, and then denatured by heating to 100° while suspended in about 5 liters of water. The denatured protein was filtered off on cheesecloth, dried, and analyzed for nitrogen.

TABLE II

PROTEIN RECOVERED FROM FLOURS BEFORE AND AFTER EXTRACTION WITH PETROLEUM ETHER

Material	Protein present	Recovery of protein present in	
		Natural flour	Extracted flour
	%	%	%
Flour A	15.0	65	53
Flour B	10.4	0	44
Flour A, 67% + 33% starch	10.4	17	41
Flour B, extracted also with alcohol-ether	10.4	—	43
Flour B, 1.4 times the usual concentration in suspension[1]	10.4	20	—
Flour C	6.5	0	44
Flour A, 43% + 57% starch	6.5	0	30
Whole (hard) wheat meal	16.2	37.5	42
Feed middlings	12.6	0	10

[1] 2800 g flour in 6.6 liters of sulfite solution.

Table II shows the percentage of the total flour protein that was recovered by this means. Because of the difficulty in handling small batches of gluten, especially when the clot is granular (as is the case sometimes with sulfite solutions), no great degree of accuracy can be claimed for the yield values. Repeated experiments not reported here indicated that these values are only reproducible to about ±5% of the flour used; that is to say, two independent measurements will vary at most by about 10%, without the variation being considered significant here. However, the flour lipids are in some way connected with such spectacular changes in the yield of protein that a greater accuracy of measurements is hardly needed to demonstrate them, even if it were practicable.

Discussion

It may be seen that the fraction of protein recovered was increased from nothing to about 40% by removing the extractable lipids from the soft wheat flours. But the yield of protein recovered never rose to the

level of that from the hard wheat flour under the conditions of these experiments. The amount of protein recovered from hard wheat was not greatly affected by removal of the extractable lipids.

When the lipids removed by petroleum ether (Table III) were reincorporated with extracted soft wheat flour, the yield of protein recovered was greatly lessened. As little as one-quarter of the lipid

TABLE III

EFFECT OF ADDED LIPIDS ON YIELD OF PROTEIN FROM FLOUR EXTRACTED WITH PETROLEUM ETHER

Material	Protein present	Total lipid	Protein recovered	
			Added lipid from same flour	Added lipid from the other flour
	%	%	%	%
Flour A, no lipid added	15.0	0.3	53	—
Flour A, flour lipid added	15.0	0.8	61	—
Flour A, flour lipid added	15.0	1.3	58	56
Flour A, flour lipid added	15.0	1.8	41	—
Flour A, flour lipid added	15.0	2.3	0	8
Flour A, cottonseed oil added	15.0	2.3	0	—
Flour A, water extract from an equal weight of flour B added	15.0	1.3	45	—
Flour B, no lipid added	10.4	0.3	44	—
Flour B, flour lipid added	10.4	0.6	16	32
Flour B, flour lipid added	10.4	0.8	0	—
Flour B, flour lipid added	10.4	1.3	0	0
Flour B, flour lipid added	10.4	1.8	0	—

material originally extracted reduced the quantity of recoverable protein to one-third of its former value. In the case of the hard wheat flour, however, the addition of 1.5 times the quantity of lipid material originally extracted was required to reduce the yield of protein to two-thirds.

The foregoing experiments show that the proportionate yield of coagulable protein from a soft wheat flour could be increased by removing part of the lipids. It could also be enlarged by increasing the concentration of protein present without changing the lipid-protein ratio. This was done by reducing the amount of liquid used with a low protein flour (B) so that the concentration of protein was the same as that for the high protein flour in the larger volume. By this means the yield of protein from flour B was increased (Table II), but not enough to equal the yield from flour A. Furthermore, mixtures of wheat starch and high protein flour (A) were made so that the protein of the mixtures corresponded to that found in flours B and C, respectively. These mixtures behaved very like the natural flours of the same (low) protein content (Table II).

There is thus a great difference in the extent to which small quantities of easily extracted flour lipids affect the yield of gluten from hard and soft wheat flour.[6] A difference in the composition of the fatty material extracted from soft wheat flour is not responsible for the low yield of protein therefrom, because this yield is equally depressed by the material extracted from hard wheat flour. A similar method of procedure was used by Finney (1943) to show that the difference in baking quality between Kharkof and Chiefkan wheat was not due to the specific nature of the extractable lipids. In considering the yield of coagulated gluten, it seems clear that the amount of lipid present in the flour is a significant factor, but the critical amount varies with the type of flour.

On the other hand, there is no evidence that the proteins in the two types of flour are responsible for their different behaviors toward sulfite solution. The addition of the water-soluble proteins from soft wheat flours had little influence on the amount of protein recovered from hard wheat flour (Table III), showing that this fraction of the soft wheat did not carry with it an anticoagulating factor sufficient to account for the differences observed between the two flours. This viewpoint is supported by the observation (Table II) that when the concentration of protein in high-protein flour was reduced by the addition of wheat starch, the mixture behaved like a flour of naturally low-protein content. But the ratio of fat to protein in the case of these artificial mixtures is not the same as for low-protein flour; on the contrary, it is the same as in the original high-protein flour. This indicates that the lipid-to-protein ratio does not alone determine the yield of protein. Flour A tolerated about twice as much lipid to protein as flour B before the yield of protein vanished.

Because of the ease with which some of the lipid material may be removed by solvent from natural flour, it seems reasonable that such lipids exist either free or on the surfaces of protein particles. In each case an accumulation of lipids at the surface of the protein can be expected when the flour is mixed with water, because so much of the fatty material, whether naturally present or artificially added, ultimately appears in the gluten. It is probable, therefore, that the proportion of easily extractable lipid to protein indicates the superficial concentration of lipids on the protein particles while the latter are still small. The higher this superficial concentration of lipids, the lower will be the number of collisions that "stick" during the churning. On

[6] If the lipid material remaining in the flour after simple extraction were responsible for the observed differences, it is difficult to understand why the yields of protein from extracted soft wheat flour were so high, and so readily lowered by returning a trace of the extracted material to the flour. Furthermore, as shown in Table II, the soft wheat flour gave the same yield of coagulated protein after extraction with both petroleum ether and alcohol-ether as it gave after extraction with petroleum ether alone, showing that the residual lipoproteins played only a minor role in the coagulation of the protein.

the other hand, the total number of collisions between protein particles must depend on their concentration, and that is roughly represented at the start by the protein content of the flour. An increase in the protein content of the flour should therefore increase the number of collisions, while an increase in the proportion of extractable lipid to protein should decrease their individual effectiveness. If, as seems likely, the surface of the protein particles is the critical area involved, soft wheat flour may be expected to give lower yields of protein.

Several factors not presently determinable would have to be known before drawing any quantitative picture from these or similar data. The sizes of the protein particles during the essential steps of the coagulation would have to be known, and also the fate of the surface lipid during that period, how much is buried in the coagulum, how much is concentrated on the new (and smaller) surface, and how much is given up to the surrounding medium. Furthermore, while the present data are too approximate to indicate it, some involvement of the chemical properties of both the lipids and proteins present is of course to be anticipated. On the other hand, if this hypothesis is assumed in principle, it must be admitted that the hard wheat flour has behaved in these experiments as though its protein particles were on the average decidedly smaller during the coagulation process than those of the soft wheat flour.

Summary

Three patent flours, one from hard wheat and two from soft wheats, were extracted with petroleum ether. The removal of lipids from soft wheat flour greatly increased the amount of coagulable protein that could be recovered on treatment with sulfite solution. The removal of lipids from hard wheat flour did not alter the yield of protein therefrom.

The addition of the flour lipids to previously extracted flour resulted in a smaller yield of protein, regardless of the type of flour from which the lipids were prepared. The amount of artificially added lipid necessary to reduce the yield of protein materially was very small in the case of the soft wheat flour; two or three times as much was required to produce a commensurate decrease in the yield of protein from hard wheat flour.

An "anticoagulating" action sufficient to explain the results could not be demonstrated in either the lipid or the protein fractions of the soft wheat flour.

The results suggest an explanation based on the hypothesis that the coherence of protein particles to form a coagulum (and possibly also a dough) varies directly with the number of the protein particles and inversely with the surface concentration of lipids thereon.

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Alpha-Tocopherol Acetate

Biotin

PURE VITAMINS

—products of Merck Research

Merck research has been directly responsible for many important contributions to the synthesis, development, and large-scale production of individual vitamin factors in pure form.

In a number of instances, the pure vitamins may be considered to be products of Merck research. Several were originally synthesized in the Merck Research Laboratories, and others have been synthesized by Merck chemists and collaborators in associated laboratories.

Because most of the known vitamins have now been made available in pure form, effective therapy of specific vitamin deficiencies can be conducted on a rational and controlled basis, under the direction of the physician.

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